Volume 23 November 1959 No. 6

BULLETIN OF THE AGRICULTURAL CHEMICAL SOCIETY OF JAPAN

ANALYTICAL CHEMISTRY ORGANIC CHEMISTRY BIOLOGICAL CHEMISTRY
FOOD & NUTRITION MICROBIOLOGY & FERMENTATION INDUSTRY
SOIL & FERTILIZER

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Published by the Agricultural Chemical Society of Japan
c/o Faculty of Agriculture, University of Tokyo

The Bulletin of the Agricultural Chemical Society of Japan is published bimonthly to provide original papers on agricultural chemistry.

Manuscripts submitted for publication and all inquiries regarding them should be sent to the Editor of the Bulletin, The Agricultural Chemical Society of Japan, c/o Departments of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

Annual subscription price is $6.00 for all oversea subscribers including postage fee. Please address order to the agencies.

The Agricultural Chemical Society of Japan also published The Journal of the Agricultural Chemical Society of Japan (Nippon Nôgei-Kagaku Kaishi), printed in Japanese.

AGENCIES

Charles E. Tuttle Co.
Rutlant, Vt.
U.S.A.

Maruzen Co., Ltd.
P. O. Box 605, Tokyo Central
Tokyo, Japan.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 6, p. 457~459, 1959]

Nematocidal Activities of Halogenoalkylcarboxylic Acid Esters

By Katsura MUNAKATA, Akio HARADA and Tsutomu NISHIZAWA*

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Received January 30, 1959

About 40 halogenoalkylcarboxylic acid esters were synthesized and their nematocidal activities were tested against the rice white tip nematode, *Aphelencoides besseyi*.

Allyl esters of halogenoacetic acids were found to inhibit completely the growth of nematode at the 8 p.p.m. level, and the most effective one was allyl bromoacetate inhibiting at the 2 p.p.m. level, while sodium N-methyldithiocarbamate, a commercial nematocide inhibited at the 16 p.p.m. level.

The relationship of variation of the acid and alcohol moieties of esters to the nematocidal activity was discussed.

The relationship of the chemical structure and the nematocidal activity is very obscure, and most of commercial nemotocides are rather harmful for plants.

For the study to find out new herbicidal chemicals, the authors had synthesized about 120 esters of halogenoalkylcarboxylic acids. As these compounds had shown various extents of herbicidal activities, the idea to test these compounds against nematodes occured. In this paper a preliminary investigation to evaluate the activity of selected 37 esters against the rice white tip nematode, *Aphelencoides besseyi*, is reported.

Geltler et al.[1] also reported about screening tests on 53 bromoacetic acid esters as nematocides of Rhabditis sp. and Panagrellus sp., and octyl, 4-bicyclohexylyl, heptyl, decyl, 4-*sec*-butylcyclohexyl and hexyl esters were shown to be effective at the 2 p.p.m. level.

METHODS AND RESULTS

The esters were prepared by the reaction of alcohols or phenols with the appropriate acids using sulfuric acid or hydrogen chloride gas as catalyst, and in other case alcohols or phenols were reacted with appropriate acid halides in pyridine.

The vinyl esters were prepared by the reaction of acids and acetylene gas according to Wileys method[2].

The chemical and physical properties of these compound will be reported separately with other compounds of this group.

All compounds to be tested were formulated in an aquerous preparations, and those insoluble in water were suitably dispersed or emulsified.

Furthermore, several nonhalogenocarboxylic acid esters, halogenoacids, alcohols, phenols and a commercial nematocide, sodium N-methyldithiocarbamate (Vapum), were also tested for comparison.

The evaluation of compounds against the nematode was carried as follows[3].

One hundred rice white tip nematodes cultivated on artificial medium[4] were poured with 3 ml of a definite concentration of the liquid preparations of esters, and kept at 27°C for 24 hours, and the lethal percentage was measured. The test was triplicated.

The results of evaluations are shown in Table I, and the figures in Table are the lethal percentage, 100 showing complete kill of the nematode and zero showing complete lack of nematocidal activity.

DISCUSSIONS

From the result of test shown in the Table I, several facts were evident.

* This report was presented for the Meeting of the Agricultural Chemical Society of Japan at Kyôto University, May, 1958.

1) S. L. Gertler, J. Feldmesser and R. V. Rebois, *J. Agri. Food Chem.*, **6**, 843 (1958).

2) R.H. Wiley, *Org. Synth.*, **28**, 94 (1948).

3) K. Iyatomi, "Shin-Noyaku Kenkuhô," Nankôdô, Co., 1958 p. 465.

4) K. Iyatomi and T. Nishizawa, *Jap. Jour. Appl. Zool.*, **19**, 8, (1954).

TABLE I. NEMATOCIDAL ACTIVIES OF HALOGENOALKYLCARBOXYLIC ACID ESTERS

Conc. in p.p.m.	256	128	64	32	16	8	4	2
Methyl chloroacetate	100	100	100	100	75	5	0	—
Ethyl 〃	100	100	100	98	43	3	0	—
Propyl 〃	100	100	100	100	95	30	0	—
Isopropyl 〃	100	100	50	13	3	0	0	—
Isobutyl 〃	100	100	90	0	0	0	0	—
Vinyl 〃	75	5	0	0	0	0	0	—
Allyl 〃	100	100	100	100	100	100	70	—
Furfuryl 〃	100	100	51	5	0	0	0	—
Tetrahydrofurfuryl 〃	100	90	22	5	0	0	0	—
Benzyl 〃	100	66	56	45	23	16	12	—
Phenyl 〃	100	100	100	52	—	—	—	—
o-Chlorophenyl 〃	30	15	3	0	0	0	0	—
m-Chlorophenyl 〃	100	100	100	100	52	30	—	—
p-Chlorophenyl 〃	100	100	0	0	0	0	0	0
2,4-Dichlorophenyl 〃	100	60	7	0	0	0	0	—
2,4,5-Trichlorophenyl 〃	100	100	100	100	78	30	0	—
2,4,6-Trichlorophenyl 〃	100	100	60	25	9	0	0	—
2-Methyl-4-chlorophenyl 〃	100	100	63	26	8	0	0	—
p-Bromophenyl 〃	100	100	100	100	95	85	65	—
p-Iodophenyl 〃	0	0	0	0	0	0	0	—
p-Nitrophenyl 〃	0	0	0	0	0	0	0	—
Butoxyethyl 〃	100	100	100	100	63	0	0	—
Allyl bromoacetate	100	100	100	100	100	100	100	99
Benzyl 〃	100	100	100	100	100	100	90	—
Butoxyethyl 〃	100	100	100	100	100	100	96	10
Allyl iodoacetate 〃	100	100	100	100	100	100	83	—
Benzyl 〃	100	100	100	100	100	100	96	—
Butoxyethyl 〃	100	100	100	100	100	100	69	—
Allyl 1,1-dichloro-propionate	100	100	98	61	34	0	0	—
Allyl 2,3-dichloro-isobutyrate	100	100	95	74	43	37	0	—
Benzyl 〃	100	83	72	68	50	14	0	—
Butoxyethyl 〃	100	99	80	47	43	24	0	—
Allyl trichloroacetate	100	55	8	0	0	0	0	—
Allyl acetate	100	100	100	100	90	26	0	—
Benzyl 〃	100	0	0	0	0	0	0	—
Butoxyethyl 〃	0	0	0	0	0	0	0	—
Sodium acetate	0	0	0	0	0	0	0	—
Sodium chloroacetate	16	0	0	0	0	0	0	—
Sodium bromoacetate	74	8	0	0	0	0	0	—
Sodium iodoacetate	100	12	0	0	0	0	0	—
Sodium methyldithiocarbamate	100	100	100	100	100	44	0	—
Allyl alcohol	100	100	100	60	26	0	0	—
Benzyl alcohol	0	0	0	0	0	0	0	—
Butoxyethyl alcohol	0	0	0	0	0	0	0	—
Phenol	0	0	0	0	0	0	0	—
o-Chlorophenol	0	0	0	0	0	0	0	—
m-Chlorophenol	8	0	0	0	0	0	0	—
p-Chlorophenol	27	0	0	0	0	0	0	—
2,4-Dichlorophenol	70	0	0	0	0	0	0	—
2,4,5-Trichlorophenol	100	90	28	0	—	—	—	—
2,4,6-Trichlorophenol	7	5	3	0	—	—	—	—
Pentachlorophenol	10	4	0	0	—	—	—	—
p-Bromophenol	40	14	3	0	—	—	—	—
p-Iodophenol	0	0	0	0	—	—	—	—
p-Nitrophenol	17	0	0	0	—	—	—	—
p-Cresole	0	0	0	0	—	0	0	0

In the case of chloroacetates, the variation in alcohol moiety of esters gave some effects for namatocidal activities, and allyl moiety showed the strongest activity.

It is worthy to note that also allyl alcohol itself showed rather strong activity, while aryl moiety such as benzyl, phenyl or substituted phenyl alcohols did not show so much increase in activities.

About the acid moiety of esters, the strongest activity was found in the case of bromoacetic acid, and the extreme strong activity was showed also by allyl bromoacetate.

The activity did not increase with the length of the carboxylic acid chain as in the case of propionic acid or iso butyric acid. From the results on sodium acetate, allyl alcohol and allyl acetate, it is clear that allyl acetate showed the strongest activity.

Accordingly, the formation of ester may contribute to the strengthening of activity.

The esters containing bromine atom which showed strong activities included *p*-bromophenyl chloroacetate, and allyl, benzyl or butoxyethyl bromoacetate.

The phenols did not show so strong nematoacidal activities in spite of their strong activities against microorganisms.

Further experiments are being conducted about nematocidal activities of related compounds, and about the toxicity for other nematodes.

Acknowledgment The authors wish to thank Prof. Y. Sumiki of the University of Tokyo for his advice and encouragement, Prof T. Tamura and Prof. K. Iyatomi for their helpful discussions, and Miss N. Ôkubo for her technical assistance in nematocide screening tests.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 6, p. 460~464, 1959]

Studies on Some New Azoxy Glycosides of *Cycas revoluta* Thunb.

Part I. On Neocycasin A, β-Laminaribiosyloxyazoxymethane*

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Received February 23, 1959

The isolation of a new glycoside, named here as neocycasin A, with use of carbon chromatography, is described. It is one of a series of aliphatic azoxy glycosides, found in the seeds of Japanese cycad together with cycasin which is β-glucosyloxyazoxymethane as reported previously. The glycoside monohydrate gives m.p. 162°~163° (decomp.), $[\alpha]_D^{29}$ −35.1°; its heptaacetylate, m.p. 142°~143°, $[\alpha]_D^{29}$ −55.5°, from which octaacetyl-β-laminaribiose is isolated. On the basis of examination of the products obtained from partial or complete hydrolysis, and spectroscopic measurements, neocycasin A is concluded to be β-laminaribiosyloxyazoxymethane, i.e. 3-O-β-D-glucopyranosylcycasin.

In another series of this paper it was reported that cycasin, a new toxic glycoside isolated from the seeds of *Cycas revoluta* Thunb., is β-glucosyloxyazoxymethane[1], and that the behaviors of its aglycone which is scarcely found in nature are also interesting both enzymatically[2] and polarographically[3].

In Australian cycads of genus *Macrozamia*, macrozamin was found by J.M. Cooper[4] and later, also found in *Bowenia* or *Cycas*[5] and its structure was proved to be β-primeverosyloxyazoxymethane[6] unlike cycasin in the sugar component. However, macrozain could not be detected in Japanese cycad in earlier studies.

During the course of further isolation of cycasin afterwards, a series of spots which were revealed to have the same color as that of cycasin was detected on paper chromatograms. This observation suggested the presence of some azoxy glycosides besides cycasin.

The seeds obtained recently, eminently containing these glycosides, were treated for the isolation of them, in. which carbon chromatography was applied, successfully. Consequently, one of these glycosides, neocycasin A, was obtained in a crystalline state.

EXPERIMENTAL AND RESULTS

Isolation

The seeds of cycad used here were collected at the suburbs of Nasé City, Kagoshima Pref., on January, 1958.

In this trial, the previous procedures for cycasin[1] were conveniently modified. The seeds, 10 kg, were hulled, 6.25 kg of kernels obtained were minced in a blendor together with ice water, then thrown immediately into about 5 l of 0.2 N sulfuric acid, by which the enzymes were inactivated and extraction was facilitated. The final volume was about 10 l, filtered through a coarse-cloth, and the residues were further extracted with three portions of 5 l of 0.1 N sulfuric acid. The completion of extraction was ascertained with a negative test for hydrocyanic acid after hot-alkali treatment of the extract. The combined extracts were neutralized to pH 6.5 with calcium carbonate, filtered, and concentrated

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* Presented before the 72nd Local Meeting of the Agricaltural Chemical Society of Japan, held at Yamaguchi, October 11, 1958.

1) K. Nishida, A. Kobayashi and T. Nagahama, This Bulletin, **19**, 77 (1955).

2) K. Nishida, A. Kobayashi and T. Nagahama, *ibid.*, **19**, 172 (1955).

3) K. Nishida, A. Kobayashi, T. Nagahama and J. Nawata, *Mem. Fac. Agr. Kagoshima Univ.*, Vol. III, p. 6 (1957).

4) J.M. Cooper, *Proc. Roy. Soc. N.S.W.*, **74**, 450 (1940). (*C.A.* **35**, 4917 (1941)).

5) N.V. Riggs, *Australian J. Chem.*, **7**, 123 (1954). (*C.A.*, **48**, 6510 (1954)).

6) B.W. Langley, B. Lythgoe and N.V. Riggs, *J. Chem. Soc.*, **1951**, 2309.

TABLE I. GLYCOSIDES AND SUGARS IN EACH ELUATE FROM THE CARBON COLUMN FOUND BY PAPER CHROMATOGRAPHY

Spots							R_F		colors revealed with reagents	
									R.	A.
±	+	++	++	++	+		.55	cycasin	y	—
++	+						.46	fructose	rb	pb
					+	±	.40	A_1	y	—
++	++	+	+				.35	glucose	—	b
					+	++	.33	A_2 (neocycasin A)	y	—
					+	±	.27	A_3	y	—
+	++	+	+	±			.23	sucrose	rb	b
					++	±	.20	A_4	y	—
			+	++	+		.19	L(laminaribiose)	—	b
					±	±		tailing A_x	y	—
					+		.11		rb	—
		+					.10		—	b
					+		.07		rb	—
+						±	.00		rb	b
a	b	c	d	e	f	g				

Eluates

Filter Paper: Tôyô No. 2. Solvent: *n*-BuOH : AcOH : H_2O (4 : 1 : 1).
Development: multiple ascending (2 runs). Reagents: R, Resorcin-HCl EtOH soln. A, Anilline hydrogen phthalate BuOH soln.
Eluates: a, with 4.7 l H_2O. b, 6.4 l followed after a. c, 7.2 l after b. d, with 3.2 l 2% EtOH. e, 4.0 l 5% EtOH. f, 5.0 l 10% EtOH. g, 4.0 l 40% EtOH.
Colors: b, brown. y, yellow. r, reddish. p, pale.

under reduced pressure at 50° to a thick syrup, 500ml.

The syrup was treated with 5 volumes of methanol to precipitate the gummy impurities, which were redissolved in their own volume of water and treated with methanol as above, repeatedly. The methanolic supernatants were concentrated and treated with lead acetate and hydrogen sulfide as usual. The filtrate was concentrated to syrup (Syrup I, 450 ml).

In order to separate the glycosides, Syrup I was chromatographied on a carbon column (300 g, dia. 5 × 42 cm). Water, and then 2,5,10, and 40% aqueous ethanol was successively passed through the column. The composition of carbohydrates in each eluate was examined by paper chromatography. As represented in Table I, the spot A group, assumed to be azoxy glycosides, in 10 and 40% ethanolic eluates was exceedingly characteristic. The last fraction which contained the substance visualized as spot A_2 alone, comparatively, was carefully concentrated to syrup. The syrup was treated with ethanol, set aside in a refrigerator, and resulted in crystallizing. After recrystallization from aqueous ethanol or methylcellosolve, 1.6 g of a colorless fine needle crystal (Fig. 1), neocycasin A, was obtained.

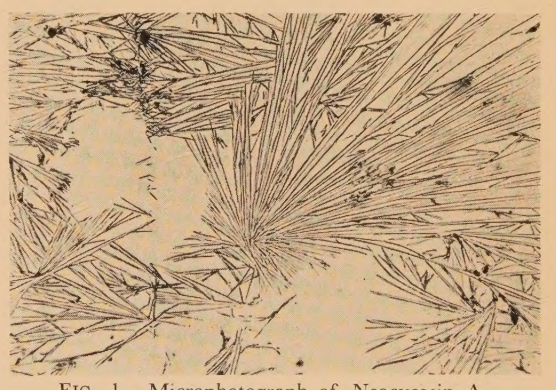


FIG. 1. Microphotograph of Neocycasin A.

Properties of Neocycasin A

Neocycasin A thus obtained showed m.p. 162°~163° (decomp.) and $[\alpha]_D^{29} - 35.1°$ (c. 1.0 in water). *Anal.* Found: C, 38.59, 38.78; H, 6.76, 6.55; N, 6.31. Calcd. for $C_{14}H_{26}O_{12}N_2 \cdot H_2O$: C, 38.88; H, 6.52; N, 6.48%. MW, Found: 411 (cryoscopic), Calcd: 432. It was soluble in hot aqueous ethanol or methycellosolve, readily soluble in water, sparklingly in ethylacetate, and

not soluble in chloroform. It gave a positive Fehling or Molish reaction and a greenish blue coloration with anthrone. On paper chromatograms it gave a single spot yellowish colored with a resorcin-hydrochloric acid reagent.

Acetylate of Neocycasin A

A solution of 500 mg of neocycasin A in 10 ml of pyridine was treated with acetic anhydride at room temperature as usual. The resultant acetylate, recrystallized from ethanol, was long prisms (FIG. 2), 540 mg, and m.p. 142°~143°, $[\alpha]_D^{29}$ −55.5° (c. 1.0 in chloroform), *Anal.* Found: C, 47.76; H, 5.85; N, 4.35. Calcd. for $C_{14}H_{19}O_{12}N_2$-$(CH_3CO)_7$: C, 47.46; H, 5.69; N, 3.95%. MW, Found: 711 (Rast), Calcd.: 708. CH_3CO, Found: 6.8 mols. per mol. of acetylate.

Deacetylated with one drop of 0.5N sodium methylate in 3 ml of ice cold chloroform-methanol, 98 mg of the heptaacetylate was reconverted into the original glycoside, 15 mg.

FIG. 2. Microphotograph of Heptaacetyl Neocycasin A.

Acid hydrolysis

1) Identification of glucose and formaldehyde in the complete acid hydrolysate With 1N hydrochloric acid, 97 mg of neocycasin A was hydrolysed at 100° for 2.5 hours. The hydrolysate being vacuum evaporated repeatedly in order to expell the produced formaldehyde, glucose in the remains was determined by the Hanes method and found to be 2.0 mols. per mol. of the glycoside. In the above distillate, formaldehyde was identified as crystalline formaldomedone.

During these hydrolysis, it was shown paper chromatographically that the spot of neocycasin A was only substituted by glucose. The phenylosazone prepared from the hydrolysate as usual, was a yellow needle, m.p. 203°~204° (decomp.) alone or on admixture with authentic glucose-phenylosazone.

2) Cycasin and laminaribose as the products of partial acid hydrolysis Neocycasin A was hydrolysed partially with 0.2 N sulfuric acid at 100° for two hours. In this partial hydrolysate were detected four spots, three of which were identified to be cycasin, glucose, and unaffected neocycasin A, respectively. The remaining one, spot L, was, according to the literature[7], closely adjacent in R_F value to that of laminaribiose or nigerose. After multiple development (3 runs) being applied in a mixture of *n*-butanol-pyridine-water (3:2:1.5 by vol.), spot L was found to be identical with that of authentic laminaribiose.

Degradation with emulsin

Degradation of neocycasin A with cycad emulsin[2] was examined in acetate buffer, pH 4.6, at 30°. Thereby, the glycoside was finally split into glucose, which was proved paper chromatographically. On the other hand, this emulsin preparation did not act on maltose under the same condition.

Action of alkali

1) Isolation of octaacetyl-β-laminaribiose from heptaacetyl neocycasin A A solution of 250 mg of heptaacetylate in 5 ml of chloroform with 2 ml of 2N methanolic sodium methylate, was kept for one hour at room temperature. The white substance, 140 mg, which precipitated with proceeding of the reaction, was collected, washed with a small amount of cold chloroform, and dried. About the contamination with some glucose due to an over reaction, it was chromatographied on carbon column (dia. 1.8×2.5 cm). After glucose was washed out with a sufficient amount of water, the fraction which was eluted with 35% ethanol was evaporated to dryness. The white powder obtained, 65 mg, showing a single spot corresponding to laminaribiose, was treated with sodium acetate and acetic anhydride at 110°~120°, and the resultant mixture was extracted with chloroform. After expulsion of the solvent, the syrup was treated with ethanol and subsequently evaporated in vacuo, repeatedly, and crystallized.

The needles, recrystallized from ethanol, were 13 mg in yield, m.p. 159°~160°, which accorded with that of octaacetyl-β-laminaribiose described in the general references. *Anal.* Found: C, 49.39; H, 5.70. Calcd. for $C_{28}H_{38}O_{19}$: C, 49.56; H, 5.64%.

2) Detection of formaldehyde and hydrocyanic acid In the above alkaline reaction mixture, the presence of formaldehyde and hydrocyanic acid which were derived from the aglycone was proved; that is formaldomedone

7) K. Aso and F. Yamauchi, *J. Ferment. Technol. Japan*, **33**, 194 (1955).

as for the former, m.p. 183°～184° alone or an admixture, and prussian blue or benzidine-cupric acetate reaction as for the latter.

Spectroscopic measurements

The ultraviolet extinction curve of neocycasin A is shown in Fig. 3 along with that of cycasin for reference.

Its infrared absorption spectrum presented a similar feature, which was characterized by a strong band near 1537 cm^{-1}, to cycasin except the band at ca. 1640 cm^{-1} as shown in Fig. 4.

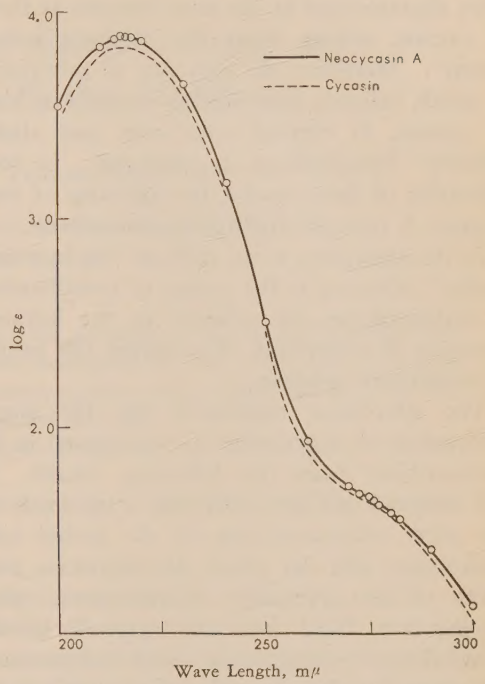


FIG. 3. Ultraviolet Absorption Spectra

Samples were examined as aqueous solution.

DISCUSSION

In the traditional methods of preparation of cycasin, the glycosidases were inactivated by boiling treatment, where the mash of boiled seeds had to be air-dried since immediate extraction was very difficult for filtration. All of these difficulties were overcome by the adoption of acid treatment. Because the stabilities of cycasin under such conditions are known[8], this modified process is at the present, also considered to be the most convenient method for similar azoxy glycosides.

Acetylation of neocycasin A with acetic anhydride and pyridine gave the heptaacetyl derivative, which was reconverted into the original glycoside with careful deacetylation.

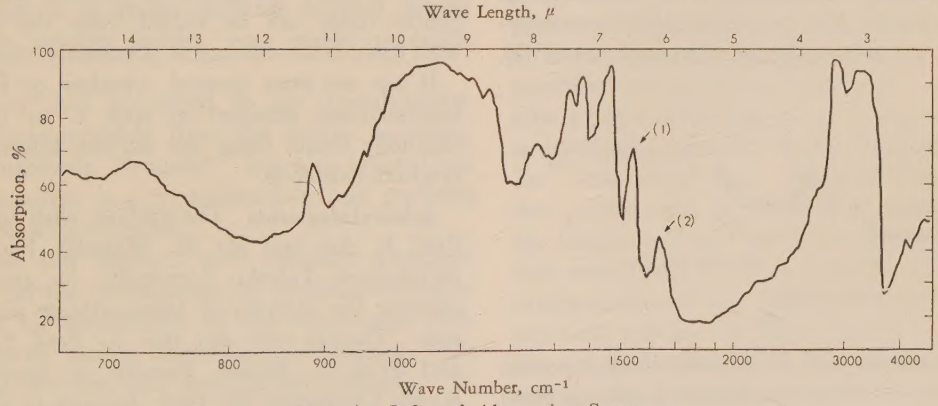


FIG. 4. Infrared Absorption Spectrum

Sample was examined as paste with nujol. The band (1) and (2) are due to the aliphatic azoxy group and the water of crystallization, respectively.

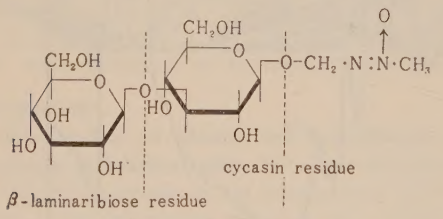
It was proved quantitatively and qualitatively that one molecule of neocycasin A liberates 2 mols. of glucose by complete hydrolysis with hydrochloric acid and also by the action of emulsin. Furthermore, in the partial acid hydrolysate, as cycasin, glucose, and a glucobiose were detected, its structure might be suggested to be β-glucosylcycasin.

The ultraviolet and infrared absorption spectra

were characterized by the same features as those of cycasin arising from the aliphatic azoxy group[1]. Moreover, the aglycone of neocycasin A which behaves in a similar manner to that of cycasin, is cleaved with acid and alkali, whereby formaldehyde is produced. In consideration of these results, the aglycone of neocycasin A must be hydroxyazoxymethane.

As the absorption at ca. 1640 cm^{-1} is, in recent studies[8], allocated to the waterc of rystallization in carbohydrates, its presence in the infrared spectrum of neocycasin A endorses the results of elementary analysis.

The glucobiose responsible for the sugar component of neocycasin A was proved to be laminaribiose from the following results. It was detected and identified with a specimen on the paper chromatograms of the partial acid hydrolysate and the alkali decomposition products of the gycloside. Furthermore, after having been freed from the aglycone by the deacetylation procedure at room temperature, the sugar from heptaacetylate was identified by derivation to a crystalline specimen, octaacetyl-β-laminaribiose. Accordingly, it is concluded that neocycasin A is β-laminaribiosyloxyazoxymethane, i.e. 3-O-β-D-glucopyranosylcycasin (I).



(I)

Laminaribiose was first obtained as a partial hydrolysate of the polysaccharide laminarin from seaweeds[9], but it has not yet been reported to exist either as glycosidic components or in a free-state in nature. As laminaribiose has recently been found in the fermented mash of barley in the Amylo process, it was proved to arise from 1,3-β-linked polyglucosan found in barley[10]. Preparation of this glucobiose by acid reversion[11,12] or enzymatic transfer of glucose[13,14] was also reported.

Possibilities of such a conversion would be denied here, because even though the acid employed in the above-mentioned procedures was sufficient to inactivate the transferring enzymes, it was unable to cause an acid reversion in which the condition of acid concentration and temperature is far more severe. Besides above, since the spots of neocycasin A and others are detected in the state of Syrup I prepared even by the traditional boiling method, these glycosides should be the components of cycad seeds, itself.

It would be responsible for no detection of such spot A group hitherto, that relative contents of the glycosides and sugars in raw seeds might vary with their habitat or degree of maturity, and large spots of sugars might mask over the glycosides. It is also considered that, being strongly absorbed on carbon, these glycosides could not be eluted from the column with such dilute ethanol as previously reported[1].

It has not been proved whether or not free laminaribiose detected as spot L in the 5% ethanolic eluate from the carbon column is in original existence.

Acknowledgements The authors wish to thank Prof. K. Aso and Mr. K. Matsuda, Faculty of Agriculture, Tohoku University for generously offering the samples of laminaribiose and nigerose. Thanks are also due to Prof. S. Takei and Prof. T. Mitsui, Faculty of Agriculture, Kyoto University for their favours to perform the infrared spectroscopy and elementary analysis.

8) S. A. Barker, E. J. Bourne and D. H. Whiffen, Carbohydrate Structure. edited by D. Glick, "Methods of Biochemical Analysis" vol. III, p. 224, Interscience (1956).

9) V. C. Barry, *Sci. Proc. Roy. Dublin Soc.*, **22**, 423 (1941). (W. Pigman, "The Carbohydrates" Acad. Press Inc., N. Y., p. 511 (1957)).

10) H. Ono and M. Dazai, *Report of the Fermentation Research Institute, Japan*, No. 14, p. 51 (1957).

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[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 6, p. 465~474, 1959]

Purification of Peptidases of *Aspergillus oryzae* and Some Properties of the Purified Peptidases

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Received December 25, 1958

The purification of *Aspergillus oryzae* peptidases was attempted by the fractional precipitation with acetone, ammonium sulphate, and by starch zone electrophoresis. We, thus, achieved a great success in the separation of dipeptidase free from aminopolypeptidase and proteinase as well as in the separation of aminopolypeptidase free from dipeptidase and proteinase.

The specific activity (C_0) of the former (leucylglycine hydrolysis) was 7000 and that of the latter (leucylglycylglycine hydrolysis) 22000.

The leucylglycine dipeptidase was remarkably activated by Zn^{++}, and Co^{++}. Some other enzyme properties were also found and are discussed.

INTRODUCTION

In regard to the proteolytic enzymes, the purification of peptidases generally seems to be carried out at a later period, as compared with that of proteinases. This might be due to the fact that it is rather difficult to find proper enzyme sources having very stable peptidase activity.

Anson[1] has succeeded in the crystallization of carboxypeptidase from the bovine pancreas.

Thenceforth, Neurath[2,3] has also isolated the same enzyme in a crystalline form and reported it to be a Zn-enzyme.

Davis and Smith[4] have obtained excellent results in the purification of prolidase of swine kidney by application of the starch-zone electrophoresis besides fractional precipitation with alcohol, acetone, and ammonium sulphate.

Hill and Smith[5] have prepared an electrophoretically homogeneous solution of leucine aminopeptidase from swine kidney by means of starch-zone electrophoresis, maintaining the stability of the enzyme by Mg^{++} ion and this enzyme is said to be activated by Mg^{++} and Mn^{++} ions. Johnson[6] has obtained an electrophoretically homogeneous yeast aminopolypeptidase, making purification by fractional precipitation with acetone. This enzyme is said to be somewhat different from the dipeptidase-free aminopolypeptidase separated by Grassmann[7] and is also able to split dipeptides to a certain extent and is activated, especially in the presence of both Zn^{++} and halide ions. Ågren[8,9] has completed the purification of amino-polypeptidase from the bovine muscle and from the pylorus mucous membrane of swine stomach by means of cataphoresis and has obtained an enzyme solution with activity 100 times higher than that of the original solution. This purified enzyme solution has an isoelectric point at pH 4.6 containing almost no dipeptidase and is not activated by Mn^{++} ion.

Binkley et al.[10] have purified the peptidases

1) M. L. Anson, *J. Gen. Physiol.*, **20**, 663 (1937), *ibid.*, **20**, 777 (1937).
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3) B. L. Vallee and H. Neurath, *J. Biol. Chem.*, **217**, 253 (1955).
4) N.C. Davis and E.L. Smith, *J. Biol. Chem.*, **224**, 261 (1957).
5) R.L. Hill and E.L. Smith, *J. Biol. Chem.*, **224**, 209 (1957).
6) M. J. Johnson, *J. Biol. Chem.*, **137**, 575 (1941).
7) W. Grassmann und Dyckerhoff, *Z. Physiol. Chem.*, **179**, 41 (1928).
8, 9) G. Ågren, *C. A.*, **39**, 4897 (1945), *ibid.*, **39**, 5260 (1945).
10) F. Binkley, V. Alexander, F. E. Bell and C. Lea, *J. Biol. Chem.*, **228**, 559 (1957).

from swine kidney in a large scale and fractionated it into leucinamidase and cysteinylglycinase portions and further purification is still in progress by means of ion exchange chromatography.

At present, among the proteolytic enzymes of *Aspergillus* mold, in regard to the proteinase, the crystallized preparation was obtained from *Aspergillus oryzae* by Crewther[11], from *Aspergillus Saitoi* by Yoshida[12], from Takadiastase by Akabori et al.[13], respectively; however, in regard to the peptidases, it seems that very few data of this kind are found except that of Johnson et al.[14,15], who attempted the purification of aminopolypeptidase by means of precipitation with acetone and subsequent adsorption with $Al(OH)_3$ C_r and $Fe(OH)_3$. Therefore, our investigations were carried out under the present title. At first, we selected *Aspergillus oryzae* RO-0129 A-2 which was kindly supplied by the Applied Mycological Institute, Tokyo University as our "stock". This strain had not only higher enzyme activities of leucylglycine and leucylglycylglycine hydrolysis but was also found to have higher enzyme stabilities. And then, the purified enzyme solution obtained from fractional precipitation with acetone as well as with ammonium sulphate, was subjected to further purification process by starch zone electrophoresis.

Application of this process enabled the authors to succeed not only in the separation of dipeptidase and polypeptidase but it was also possible to increase the purification degree.

This paper was read at the Symposium of Enzyme Chemistry held at Sapporo (Hokkaido University) on July 14, 1958; Selective parts were also presented at the Congress of the Agricultural Chemical Society of Japan, (1957).

EXPERIMENTAL PART

(A) Preparation and Purification of Enzymes Solutions of *Aspergillus oryzae*.

(1) Culture of mold and extraction of enzymes.

A three per cent skim milk medium containing 3% glucose was prepared and adjusted to pH 7.0. Each 100-ml of the medium was introduced into 500-ml Erlenmeyer flasks and autoclaved (15 lbs, 15 min.) for sterilization. The spores of the mold were inoculated to the medium and cultured in a thermostat at 30°C for 7 days. Each mycelial mat grown up on the surface of each medium was harvested and the back-side was washed with water, the moisture thus adhered being absorbed with a filter paper.

The mycelial mats thus obtained were finely chopped with scissors and mixed well with water in an amount 10 times that of the quantity of dried mold substance. The mixture was subjected to homogenization (8000~10000 r.p.m.) and after being adjusted to pH 7.0, allowed to stand for extraction in a water thermostat at 30°C for 2 hours. It was then centrifuged for 10 minutes (3000 r.p.m.) and the supernatant liquid was collected as a crude enzyme extract.

(2) Fractional precipitation with acetone.

The crude enzyme extract was cooled down below 0°C and cold acetone was added to bring the final acetone concentration up to 20 per cent. The mixture was left to stand in an ice-bath for 15 minutes and then centrifuged (4000 r.p.m.).

The precipitate was collected and washed with cold acetone and dried in a vacuum desiccator over $CaCl_2$; the supernatant liquid was cooled below 0°C and its acetone concentration raised up to 40% and subsequently to 65% by further additions of cold acetone. The precipitate at each acetone concentration was collected and washed with cold acetone by treatment similar as described above and dried in vacuo. In case of the precipitate treated with 0~20% acetone, the activities of peptidases were found to be practically negligible. As for the precipitate with 20~40% acetone, activity of the leucylglycylglycine splitting enzyme occupied the main part of the enzyme activities and in case of the precipitate with 40~65% acetone, the activities of splitting various dipeptides were found to be very strong.

Thus, the specific activity C_0 of the leucylglycine splitting enzyme solution of the final precipitate was 200~230, i.e., the specific dipeptidase activity of the precipitate with 40~65% acetone was about 10 times higher as compared with the crude enzyme extract.

(3) Fractional precipitation with ammonium sulphate.

a) In case of dipeptidase, 4 g of the dried precipitate obtained by treatment with the 40~65% acetone concentration were mixed twice with each 50-ml of water,

11) W. G. Crewther, *Nature*, **165**, 680 (1950).
12) F. Yoshida, This Bulletin, **20**, 254 (1956).
13) S. Akabori, B. Hagiwara, T. Ikenaka and S. Sakota, *Symposium of Enzyme Chemistry* (Japan), **8**, 49 (1953).
14) M. J. Johnson and W. H. Peterson, *J. Biol. Chem.*, **112**, 25 (1936).
15) J. Berger and M.J. Johnson, *J. Biol. Chem.*, **130**, 641 (1939).

under well stirring and left to stand for 30 minutes at room temperature each time and then centrifuged (3000 r.p.m., 10 min.).

The supernatant liquid was cooled down below 0°C, adjusted to pH 8.0, and added ammonium sulphate (crystals) to bring the final concentration up to 40% saturation and pH 7.0, allowed to stand in an ice bath for one hour and centrifuged (12000 r.p.m., 0°C, 15 min.). The precipitate was removed to make it free from leucylglycylglycine splitting polypeptidase as much as possible and the cold supernatant liquid adjusted to pH 8.0, to which ammonium sulphate (crystals) was added to raise the final concentration up to 80% saturation, making pH 7.0, then left to stand in an ice-box for one hour and centrifuged (12000 r.p.m., 0°C, 20 min.). The precipitate was collected, dissolved in 50 ml of water and subjected to dialysis for 40 hours at 0°C under reduced pressure (ca. 120 mm. Hg).

The enzyme solution, which was concentrated to 35 ml during dialysis, was adjusted to pH 7.5, to which ammonium sulphate was added to bring the final concentration up to 50% saturation, making pH 7.0, then allowed to stand in an ice-bath for one hour and centrifuged (12000 r.p.m., 0°C, 15 min.).

The precipitate was removed to make it free from the polypeptidase as much as possible and the cold supernatant liquid was adjusted to pH 7.0, to which ammonium sulphate (crystals) was added to raise the final concentration up to 60% saturation, making pH 7.0, then left to stand in an ice-bath for one hour and centrifuged (12000 r.p.m., 0°C, 20 min.).

The precipitate was collected, dissolved in 50 ml of water and subjected to dialysis for 70 hours at 0°C, under reduced pressure. Thus, 1~2 ml of enzyme solution that concentrated during the dialysis was obtained.

Specific activity C_0 of the final leucylglycine splitting dipeptidase solution was 1250. Therefore, the value of C_0 was raised up to ca. 50 times higher, as compared with the crude enzyme extract.

b) In the case of polypeptidase, similar treatment was carried out and the fractional precipitate obtained with 40~55% saturation of ammonium sulphate was collected and dialysed.

(4) Purification by starch zone electrophoresis. For this purpose, the method of H.G. Kunkel[16] was adopted and an electrophoresis apparatus manufactured by the Tōyō-Roshi (Oriental-Filter Paper) Co. Ltd. (Japan), was employed and performed in a room at a temperature as low as 2°C (±2). The process is described in detail as below :-

Pharmacopoeian potato starch was purified by washing throughly with water and drying by successive treatment with alcohol, and ether. One hundred and twenty grams of the purified potato starch were taken into a beaker and mixed throughly with an excess of veronal buffer (sodium barbitar (veronal) barbituric buffer) of pH 8.6 and ionic strength of $\mu=0.1$, and the supernatant liquid was removed by decantation.

This process was repeated twice or thrice and then, the starch suspension was poured onto a horizontal long plastic case ($38\times4\times1$ cm^3) and left to stand for a while.

The buffer solution appearing on the upper-part of the starch block was absorbed by filter paper and a groove of 1 cm-width was digged at a starting position of the starch block.

The groove was just fully filled with the purified dry starch to which ca. 1.5~2.0 ml of the enzyme solution purified by successive fractional precipitation with acetone, and ammonium sulphate was homogeneously poured onto. An electric current (12 m amp. and ca. 320 V) was then passed through the long block of starch for 18 hours. This process was carried out as stated above, in a cold room at ca. 2°C and the temperature of the starch block was kept below 6°C, by cooling with ice placed on the plastic plate of the plastic case. The long horizontal column of the starch block was cut into many pieces at each 1 cm distance and each piece of the starch was introduced into the test tubes respectively, and extracted with each 5-ml of water by shaking well for half an hour in the ice chamber and filtered through each dry filter. With each filtrate*, enzyme activities and protein N** were determined and are illustrated in Figs. 1,2 and Fig 7.

(B) Preparation of substrates and substrate-buffer solutions.

(1) The preparation of peptides.

Glycylglycine (Gly-Gly), glycyl-L-leucine (Gly-Leu), Glycyl-DL-valine (Gly-Val), triglycine (Gly-Gly-Gly), and glycylglycyl-L-leucine (Gly-Gly-Leu), were synthesized by the phthalyl method[17]. DL-Leucylglycine (Leu-Gly), DL-alanylglycine (Ala-Gly), DL-valylglycine

16) H. G. Kunkel and A. Tiselius, *J. Gen. Physiol.*, **35**, 89 (1951).

* A portion of 0.1ml. of each filtrate was diluted to 1.0 ml with water and filtered through a dry filter. A quantity of 0.1 ml of each diluted filtrate was used as the enzyme solution in 1 ml of the digestion mixture.

** Protein N was determined by Folin's colorimetric method by means of electric photometer.

17) J. C. Sheehan and V. S. Frank, *J. Am. Chem. Soc.*, **71**, 1856 (1946); K. Yamashita and Y. Sahashi, *J. Agr. Chem. Soc. (Japan)*, **28**, 672 (1954).

(Val-Gly), DL-α-amino-butyrylglycine (But-Gly), DL-phenylalanylglycine (Phe-Gly), glycyl-DL-alanine (Gly-Ala), glycyl-L-tyrosine (Gly-Tyr), chloroacetyl-L-tyrosine (Cl-Ac-Tyr), and chloroacetyl-L-leucine (Cl-Ac-Leu), were prepared according to Fischer's method[18]. DL-Alanylglycylglycine (Ala-Gly-Gly), DL-leucylglycylglycine (Leu-Gly-Gly), DL-alanylyglycyl-L-leucine (Ala-Gly-Leu) and DL-norleucylglyclyglycine (N-Leu-Gly-Gly) were synthesized by means of both of the above methods and L-Leucinamide (Leu-NH_2) was prepared after Smith's method[19].

Benzoylargininamide hydrochroride (Bz-Arg-NH_2-HCl) and benzoylglycinamide (Bz-Gly-NH_2) were generously supplied through the courtesy of Dr. F. Yoshida of the Noda Institute for Scientific Research, to whom our hearty thanks are due.

(2) Preparation of the substrate-buffer solution.

Unles otherwise stated, a substrate buffer solution containing 0.1 M of L-peptide or 0.2 M of DL-peptide and 0.1 M of Tris (hydroxymethyl) aminomethane (as Tris-HCl buffer) per litre at pH 7.0, was prepared as usual.

A portion of 0.5 ml of the substrate buffer solution thus prepared was used in each 1 ml of the digestion mixture.

(C) Determination of peptidase activity.

(1) Method:- The digestion procedure and determination of hydrolysis of peptides were principally carried out, following the methods described in detail in our previous communications.

In the present case, however, instead of adopting our semi-micro titration, a micro-titration procedure deviced by Grassmann and Heyde[20] in accordance with the principle of Willstätter and Waldschmidt-Leitz's alcohol titration, was applied and this method was verified and proved not only to be favorable for economical use of expensive substrates but also effective to obtain accurate reproducible results.

In case of our method, a slight modification was made by employing a small test tube with two limbs respectively, as a small digestion vessel, instead of using a small measuring flask with a narrow mouth such as that which was employed in Grassmann's experiment.

The conditions of digestion, unless otherwise noted, were as follows:- pH 7.0 (0.05 M Tris), 0.05 M for L-peptide and 0.1 M for DL-peptide (0.05 M Tris), total volume of each digestion mixture 1 ml, digested at 40°C for 1 hour. Enzyme concentrations were expressed in mg of protein N per ml of the digestion mixture. Both, before and after digestion, 0.2 ml of each digestion mixture was pipetted up by the same micro-pipette from each digestion vessel respectively and poured into 2 ml of absolute alcohol to stop the enzyme action. (Each micro-pipette was cleansed, with a small amount of the same digestion mixture, just before pipetting up after digestion.

Titration was carried out with 1/100 N KOH alcohol (90%) solution, adding a few drops of 0.1% thymolphthaleine alcohol solution as the indicator. Thus,

X = increase of titration value expressed in ml of 1/100 N KOH per 0.2 ml of the digestion mixture.

It should be noted here that the total scission corresponds to 1 ml of 1/100 N KOH per 0.2 ml of the digestion mixture, as one peptide bond of each L-peptide was completely split even when the DL-peptides were used. Therefore,

$$\text{Hydrolysis per cent} = (X/\text{Total Scission}) \times 100 = (X/1.00) \times 100$$

(2) Specific activity and total enzyme units.

In order to compare the peptidase activity and the total enzyme units of each fraction obtained by the purification processes, the following calculations were made:-

(It should be noted here that the data were taken for calculation only in range of the zero-order reaction).

K_0 = Velocity constant of the zero-order reaction.
= Hydrolysis per cent/Time of digestion.
= Hydrolysis per cent per minute.

C_0 = Specific activity = Coefficient of hydrolysis
= Proteolytic coeficient = K_0/E

Where, E = mg of (enzyme) protein N per ml of each digestion mixture.

Total units of each fraction of enzyme solution = $C_0 \times$ mg of protein-N* contained in each fraction of enzymy solution.

(3) Determination of the effect of metal ions on the peptidase activity.

a) For the measurement of the effect of metal ions such as Zn, Co, Mg, Mn, etc., we prepared 1/100 M of each sulphate** solution and mixed them in each

18) E. Fisher, Untersuchungen über Aminosäuren, Polypeptide und Proteine I, II (1919).

19) E. L. Smith and N. B. Slonim, *J. Biol. Chem.*, **176**, 835 (1948).

20) W. Grassmann und W. Heyde, *Z. Physiol. Chem.*, **183**, 32 (1929).

* Protein-N was determined by either the micro-Kjeldahl method or micro-colorimetric method[21] using Folin's reagent with an electric photometer.

** In experiments of other authors, chlorides have been commonly used, perhaps being due to the pK value being somewhat higher.

21) S. Akabori, "Method in Enzymology", (JAPAN) I, 165 (1957).

digestion mixture from the beginning of the digestion experiment. Unless otherwise stated, the concentration of each metal ion in the digestion mixture was 1/2000M in usual cases.

b) For the pre-incubation test of metal ions, a mixture of enzyme (E) and metal ion without the substrate or a mixture of substrate (S) and metal ion without the enzyme was prepared before the completion of each digestion mixture and allowed to stand at 30°C for definite periods. Then, the process of digestion and the determination of hydrolysis of peptides were carried out after the same method as (a).

(4) For the determination of the reversible effect of chelating agents on the peptidase activity, EDTA or oxine was added to the enzyme solution making its final concentration 3.6 M, and left to stand at 3°C for 24 hours. We next determined the inhibitory effect (%) of each chelating agent at concentrations as those noted in Tabs. II, VIII. For the removal of each chelating agent from the above-treated mixture, the mixed solution was subjected to dialysis for 70 hours at a low temperature, below 3°C, under reduced pressure; on the other hand, we have also added equimolar Zn^{++} or Mg^{++} after treatment with EDTA or *o*-phenanthroline. Peptidase activity was thus determined and is shown in Tabs. V and X.

(5) Determination of pH-activity-curves.

A sample of 0.5 ml of each substrate buffer solution prepared as described above (2), was mixed in 1 ml of each digestion mixture. Consequently, the concentration of each buffer constituent in the digestion mixture at various pH values was as follows:-

Kind of Buffer	Conc. of Buffer*	pH
Tris	0.05 M	7.0,7.5,8.0
Veronal-HCl	0.05 M	8.0,9.0,9.2
Acetate-acetic acid	0.10 M	5.0,6.0

* The concentration of Tris, veronal or total CH_3COO^- in each digestion mixture.

EXPERIMENTAL RESULTS

Experimental results are shown in Tables I~XI and are illustrated in Figs. 1~10. Some important points are described below:—

(1) Purification and Specific Activities.

Leucylglycine dipeptidase was fractionally precipitated at 40~65% acetone concentration followed by 50~60% ammonium sulphate saturation at pH 7.0, while leucylglycylglycine splitting enzyme was fractionally precipitated with a 20~40% acetone concentration and subsequently with 40~55% ammonium sulphate saturation at pH 7.

Each peptidase fraction was dialysed with a collodion sack at a low temperature, below 5°C, under reduced pressure and each dialysed frac-

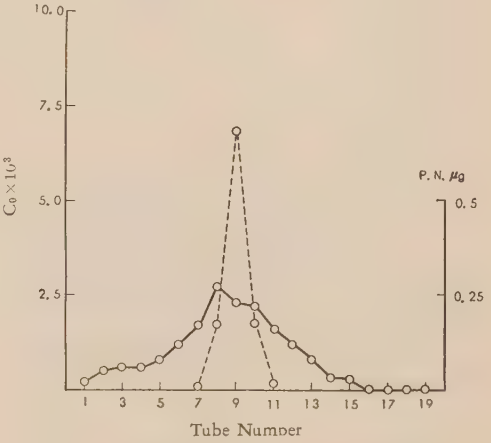

FIG. 1. Distribution of Enzyme Activities (C_0) and Protein-N along the Starch Zone by the First Electrophoretic Run at 10~12 m amp and 320 V for 18 hours.

At pH 8.6 and $\mu = 0.1$ (veronal buffer), the enzyme travelled towards the positive electrode. The dash line represents C_0 of Leu-Gly dipeptiase.

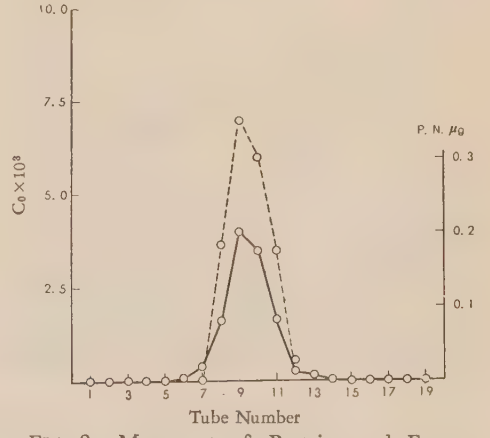

FIG. 2. Movement of Proteins and Enzyme Activities Splitting Leucylglycine by the Starch Zone Electrophoresis (2nd run).

Electrophoresis was performed for 18 hours at 10~12 m amp and 320 V at 6°C. The dash line represents C_0.

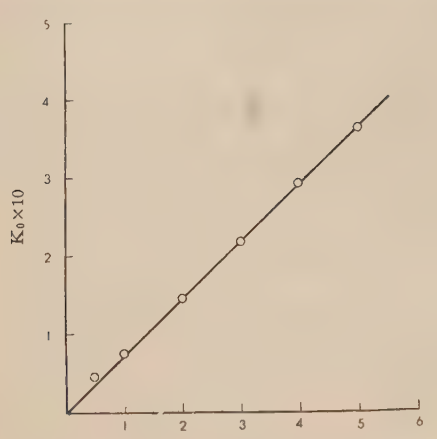

FIG. 3. Effect of Enzyme Concentration on the Velocity Constant (K_0) of Hydrolysis of Leucylglycine at pH 7.0 and 40°C.

Fraction No. 9 was used as enzyme solution and the enzyme was activated with 0.2×10^{-3} M Zn^{++} during the digestion process.

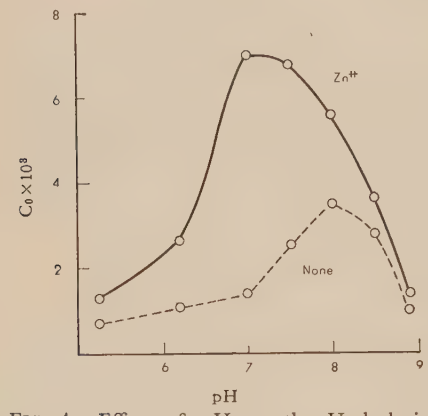

FIG. 4. Effect of pH on the Hydrolysis of Leucylglycine at 40°C with and without 0.2×10^{-3} M Zn^{++}.

Fraction No. 9 was used as enzyme which contained 0.2 μg of protein N per ml of the digestion mixture.

tion concentrated in such a manner was subjected to starch zone electro phoresis. (Figs. 1 and 7)

Fig. 2 shows the leucylglycine dipeptidase activity in the second electrophoretic run using starch zone electrophoresis and also shows an almost single peak of enzyme protein.

From this procedure, we obtained dipeptidase

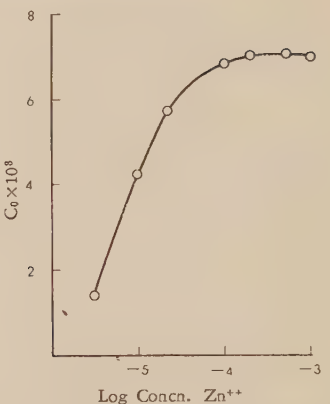

FIG. 5. Effect on Zn^{++} Concentration on the Hydrolysis of Leucylglycine at pH 7.0 and 40°C.

Fraction No. 9 was used as enzyme which contained 0.2 μg protein N per ml of the digestion mixture.

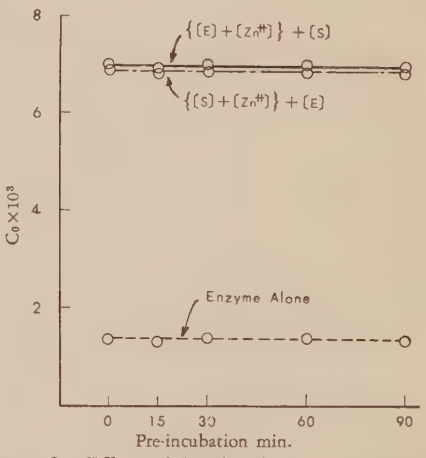

FIG. 6. Effect of Pre-incubation with Zn^{++} on the Hydrolysis of Leucylglycine.

Fraction No. 9 was used as enzyme.

{[E]+[Zn^{++}]}+[S] denotes that the substrate was added after the pre-incubation of the mixture of enzyme and Zn^{++}, and {[S]+[Zn^{++}]}+[E] denotes that the enzyme was added after the pre-incubation of the mixture of substrate and Zn^{++}.

Each pre-incubation was carried out at pH 7.0 and 30°C, with 1/600 M Zn^{++} and the enzyme activity was tested at pH 7.0 and 40°C, with 0.2×10^{-3} M Zn^{++}. The enzyme concentration corresponded to 0.2 μg of protein N per ml of the digestion mixture.

free from aminopolypeptidase and aminopolypeptidase free from dipeptidase in a highly purified condition. (Tables, I and VII)

(2) Enzymic Properties.

(a) Effect of Metal Ions. The leucylglycine

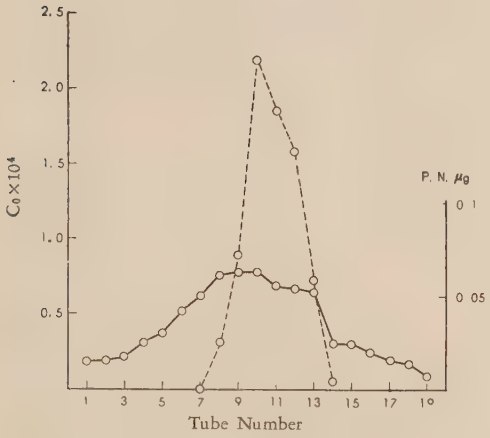

FIG. 7. Movement of Proteins and Enzyme Activities Splitting Leucylglycylglycine by the Starch Zone Electrophoresis (1st run).

Electrophoresis was performed for 18 hours at 10 m amp and 320 V at 6 C. The dash line represents C_0 of Leu-Gly-Gly.

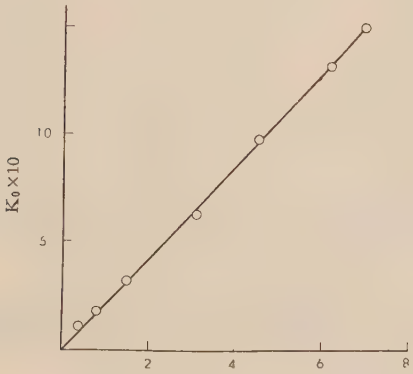

FIG. 8. Effect of Enzyme Concentration on the Velocity Constant (K_0) of Hydrolysis of Leucylglycylglycine at pH 7.0 and 40°C.

Fraction No. 10 was used as enzyme solution.

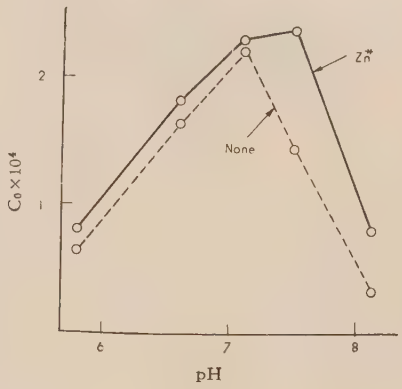

FIG. 9. Effect of pH on the Hydrolysis of Leucylglycylglycine at 40°C with and without 0.5×10^{-3} M Zn^{++}.

Fraction No. 10 was used as enzyme which contained 0.052 μg of protein N per ml of the digestion mixture.

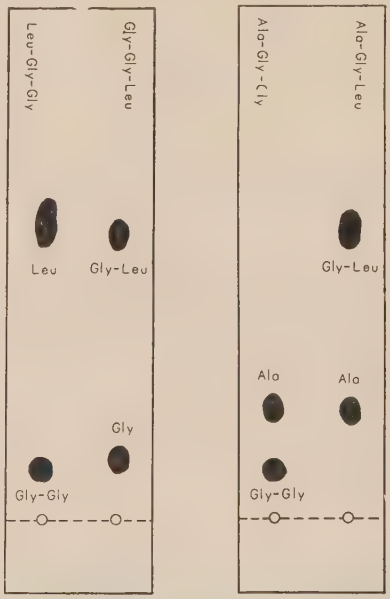

FIG. 10. Paper Chromatograms of the Enzymic Hydrolysate of Several Tripeptides.

Fraction No. 10 was used as enzyme.
Development solvent: iso-butanol, acetic acid, water (4 : 1 : 1)

splitting dipeptidase was remarkably activated by Zn^{++} and Co^{++}, the optimal concentration being 0.2×10^{-3}M, respectively, (Fig. 5) while, the leucylglycylglycine splitting enzyme was slightly effected by these metal ions, at the same concentration as above. By use of the following metal ions, inhibition was almost completed: Cd^{++}, Cu^{++}, Ag^{++} and Pb^{++}, and Mg^{++} and Mn^{++} had no activation effect.

(b) Effect on the Enzyme Concentration. The zero-order rate constant for the hydrolysis of leucylglycine and leucylglycylglycine was linear with enzyme concentration. (Figs. 3 and 8)

TABLE I. SPECIFIC ACTIVITIES (C_0), etc., OF LEUCYLGLYCINE SPLITTING DIPEPTIDASE OF EACH FRACTION OBTAINED IN THE COURSE OF PURIFICATION

Fraction	Specific activities (C_0)	Total units	Yield (%)
Crude extract	30	4650	100
Ppt obtained with 40–65% acetone concn.	200	2800	60.2
Ppt obtained with 50–60% saturation of $(NH_4)_2SO_4$	1250	2625	56.7
Enzyme soln. obtained by starch zone electrophoresis	6800	410	8.8

TABLE II. EFFECT OF METAL CHELATING AGENTS ON THE HYDROLYSIS OF LEUCYLGLYCINE

Chelating agents	M. concn.	Inhibition (%)
1,10-Phenanthroline	10^{-3}	99
	10^{-4}	92
EDTA	10^{-3}	99
	10^{-4}	93
Oxine	0.2×10^{-3}	90

Enzyme concn. corresponded to 0.2 μg protein N per ml of the digestion mixture.

TABLE III. EFFECT OF SH-REAGENT ON THE HYDROLYSIS OF LEUCYLGLYCINE

SH-reagent	M. concn.	Inhibition (%)
PCMB	10^{-4}	0
Mono-I-acetate	10^{-2}	0
Hg	10^{-4}	10
NaCN	10^{-3}	0

Enzyme concn. corresponded to 0.2 μg of protein N per ml of the digestion mixture.

TABLE IV. EFFECT OF PREVIOUS TREATMENT OF ENZYME WITH ACID OR AT HIGHER TEMPERATURE ON THE HYDROLYSIS OF LEUCYLGLYCINE

Treatment	Inhibition (%)
Treatment at pH 4.0 (0°C, 30 min)	75
Treatment at 55°C (at pH 7.0)	
〃 for 5 min	60
〃 for 10 min	86
〃 for 15 min	93

Enzyme concn. corresponded to 0.2 μg of protein N per ml of the digestion mixture.

TABLE V. REVERSIBLE EFFECT OF EDTA ON THE HYDROLYSIS OF LEUCYLGLYCINE

Treatment	Hydrolysis (per cent)	
	No addition	With 1/2000 M Zn^{++}
None	17.0	67.5
Addition of 1/2000 M EDTA	6.0	67.0
Removal of EDTA by dialysis	16.0	68.0

Enzyme soln. (specific activity $C_0 = 1250$) was used in this case.

TABLE VI. SUBSTRATE-SPECIFICITIES OF DIPEPTIDASE

Substrate	Time of digestion, hr.	Hydrolysis (per cent)		
		None	Co^{++}	Zn^{++}
Leu-Gly	0.5	8.0	24.0	43.0
Gly-Gly	2.0	4.0	26.5	—
Gly-Leu	2.0	1.0	30.0	—
Gly-Val	2.0	5.0	10.0	—
Gly-Phe	2.0	0.0	16.0	—
But-Gly	1.0	3.0	16.2	—
Phe-Gly	1.0	8.0	15.0	—
Leu-NH_2*	3.0	0	0	0
Leu-Gly-Gly	3.0	0	0	0
Ala-Gly-Gly	3.0	0	—	—
Gly-Gly-Gly	3.0	0	—	—
Gly-Gly-Leu	3.0	0	—	—
Cl-Ac-Leu	3.0	0	—	—
Cl-Ac-Tyr	3.0	0	—	—
Bz-Arg-NH_2**	6.0	0	0***	—
Bz-Gly-NH_2**	6.0	0	0***	—
Casein	6.0	0	—	—

Enzyme concn. corresponded to 0.2 μg of protein N per ml of the digestion mixture, and metal ion concn. was 1/2000 M.
* Substrate concn., 0.025 M.
** Acetate buffer, pH 5.5.
*** Addition of 0.5×10^{-3} M cysteine.

(c) Effect of pH. Leucylglycine dipeptidose showed its optimal activity at pH 8.0 in the absence of metal ions, (Fig. 4) while leucylglycylglycine splitting enzyme showed an optimal pH at about 7.0. (Fig. 9)

(d) Effect of Preincubation. The activation of leucylglycine dipeptidase was approximately neither increased nor decreased by the time-length of the previous treatment carried out with Zn^{++} and enzyme or the substrate prior to the digestion experiment. (Fig. 6)

TABLE VII. SPECIFIC ACTIVITIES (C_0), etc., OF LEUCYLGLYCYLGLYCINE SPLITTING AMINO POLYPEPTIDASE OF EACH FRACTION OBTAINED IN THE COURSE OF PURIFICATION

Fraction	Specific activity, C_0	Total units	Yield (%)
Crude extract	70	4270	100
Ppt obtained with 20–40% acetone concn.	850	1487	34.8
Ppt obtained with 40–55% saturation of $(NH_4)_2SO_4$	15800	1106	25.9
Enzyme soln. obtained by starch zone electrophoresis	22300	267	6.3

TABLE VIII. EFFECT OF VARIOUS REAGENTS ON THE AMINO POLYPEPTIDASE

Reagent	M. concn.	Inhibition of Leu-Gly-Gly (%)
EDTA	1/2500	93
	1/1000	99
o-Phenanthroline	1/2000	95
	1/1000	100
Oxine	1/2500	90
NaCN	1/2000	0
PCMB	1/5000	0
Mono-I-acetate	1/ 250	0
Cysteine	1/2000	20

Enzyme concn. corresponded to 0.062 μg of protein N per ml of the digestion mixture.

TABLE IX. EFFECT OF PREVIOUS TREATMENT OF ENZYME WITH ACID OR AT HIGHER TEMPERATURE ON THE HYDROLYSIS OF LEUCYLGLYCYLGLYCINE

Treatment	Inhibition (%)
Treatment at pH 4.0 (5°C, 30 min)	80
Treatment at 55°C (at pH 7.0)	
〃 for 5 min	16
〃 for 10 min	32.9
〃 for 15 min	47.7

Enzyme concn. corresponded to 0.062 μg of protein N per ml of the digestion mixture.

TABLE X. INHIBITION OF AMINOPOLYPEPTIDASE BY *o*-PHENANTHROLINE AND ITS REVERSIBILITY BY THE ADDITION OF METAL ION

Substrate	*o*-Phenanthroline (M)	Metal ion (M)	Hydrolysis (%)
Leu-Gly-Gly	0	0	100
〃	0.5×10^{-3}	0	4
〃	0.5×10^{-3}	Zn^{++} 0.5×10^{-3}	96
〃	0.5×10^{-3}	Mg^{++} 0.5×10^{-3}	6

Enzyme concn. corresponded to 0.062 μg of protein N per ml of the digestion mixture.

TABLE XI. SUBSTRATE-SPECIFICITIES OF AMINOPOLYPEPTIDASE

Substrate	Enzyme concn. (μg)	Time of digestion, (hr.)	Hydrolysis (%)
Leu-Gly-Gly	0.062	1	78
Gly-Gly-Leu	〃	1	61.5
Ala-Gly-Gly	〃	1	23
Ala-Gly-Leu	〃	1	58
Gly-Gly-Gly	〃	2	5
Leu-Gly	0.124	1	0
Ala-Gly	〃	1	0
Gly-Gly	〃	2	0
Gly-Leu	〃	2	0
Cl-Ac-Leu	〃	2	0
Cl-Ac-Tyr	〃	2	0
Leu-NH_2*	〃	2	0
Bz-Arg-NH_2	〃	3	0
Bz-Gly-NH_2	〃	3	0
Casein**	〃	6	0

Enzyme concn. μg, denotes the content of protein-N per ml of the digestion mixture; pH 7.0, Tris-HCl buffer; in the cases denoted*, and**, pH 8.0 Tris-HCl buffer and pH 7.0, Mc-Ilvaine buffer were used respectively.

(e) Effect of Chelating Agents. Both, leucylglycine dipeptidase and leucylglycylglycine splitting enzyme were remarkably inhibited by the chelating agents.

Inhibition by EDTA, and oxine was eliminated by dialysis and inhibltion by *o*-phenanthroline, and EDTA was removed by the addition of equimolar Zn^{++} but not by the addition of Mg^{++}. (Tables II, V, VIII and X)

(f) Effect of SH-Reagents. Both enzymes were not inhibited by the SH-reagents. (Tables III and VIII)

(g) Effect of Heating or Acid. Tables IV and IX show the effect of treatment with high temperature (55°C) or pH 4 on both enzymes.

(h) Substrate-specificities. The substrate-specificities for both leucylglycine dipeptidase and leucylglycylglycine splitting enzyme were obtained. (Tables VI and XI, Fig. 10)

CONSIDERATIONS

(1) In experiments with kidney, Smith et al.[5], by application of starch zone electrophoresis, succeeded in the separation and purification of a single enzyme fraction, leucine ami-

nopeptidase which hydrolyzes both leucylglycine and leucylglycylglycine.

Whereas, according to our experimental results, especially when Figs. 2 and 7 illustrating the starch zone electrophoresis relation and Tabs. VI, XI, etc., concerning the substrate specificities are considered, the dipeptides and tripeptides, at least in the case of Aspergillus mold, seem to be hydrolyzed by each individual enzyme. Further, in contrast with that leucine aminopeptidase of swine kidney is remarkably activated by Mn^{++}, and Mg^{++} ions, respectively, leucylglycinase of Aspergillus mold is remarkably activated by Zn^{++}, and Co^{++} ions, while, leucylglycylglycinase which originated from the same source is slightly affected by Zn^{++} ion, etc. These facts also emphasize the above considerations.

(2) From the kidney of swine, Smith et al.[4], have conducted separation of another kind of dipeptidase named prolidase and reported it to be a SH-enzyme and activated by Mn^{++} ion. Whereas, both of the peptidases of Aspergillus mold noted above do not seem to be SH-enzymes, from Tabs. III and VIII.

These peptidases might be metal enzymes, in consideration of the fact that they are remarkably inhibited by the metal chelating agents and such inhibitions are removed by either dialysis or the addition of Zn^{++} ion equimolar to the chelating agents.

Neurath et al.[3], purified carboxypeptidase by recrystallization and made an accurate determination of an extremely small quantity of Zn as its constant constituent and on the other hand, found that the inhibition of the enzyme by *o*-phenanthroline, etc., is removed by the addition of equimolar Zn^{++} and Cu^{++}, etc., enzyme activity thus being recovered. From these facts, they concluded it to be a Zn metal enzyme.

In order to exactly determine that our dipeptidase of Aspergillus mold is also a Zn or Co enzyme, a similar accurate determination and experiments with a further extremely purified enzyme preparation are considered desirable.

Acknowledgements Several species of *Aspergillus* mold were generously supplied through the courtesy of Dr. H. Iitsuka of the Institute of the Applied Mycology, University of Tokyo. Some purified amino acids which were used for the syntheses of peptides were kindly supplied through the courtesy of Dr. Y. Tsuchiya, and Dr. H. Oeda, the Director of the Institute of the Aji-no-moto Co. Ltd., to whom our hearty thanks are due. Thanks are also due to Prof. Dr. S. Funahashi and Dr. T. Kobayashi for their kinds permission to use their electrophoresis apparatus at the University of Tokyo. Finally, we wish to express our thanks to Assist. Prof. T. Ouchi and Mr. A. Hiramatsu of this laboratory, for their kind help in the preparation of some of our peptides.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 6. p. 475~481, 1959]

Microbiological Studies of *Coli-aerogenes* Bacteria
Part VII. Degradation of Citrate

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Received December 31, 1958

The cell-free extracts of *coli-aerogenes* produced glyoxylate and succinate in addition to pyruvate and α-ketoglutarate when incubation was carried out with a citrate-medium. The fresh cell-extracts possessed a very poor ability to produce α-ketoglutarate from citrate, but the addition of coenzyme II (TPN) to the reaction mixtures brought about an increase in the yield of α-ketoglutarate. The degradation of citrate by the intact (washed), dried and ground cells of the bacteria also resulted in an accumulation of glyoxylate in the external medium in which semicarbazide was present. It was suggested that the concentration of coenzyme II within the bacterial cells was relatively low, so that coenzyme II-dependent isocitric dehydrogenase was not operative at such a high rate enough to effect the complete removal of citrate. The bacterial cells revealed a high potency of producing α-ketoglutarate from pyruvate or succinate under aerobic conditions. It was indicated that the major breakdown of citrate by the cells did not proceed by way of isocitric-α-ketoglutaric dehydrogenase system, but occurred by way of splitting-reactions.

INTRODUCTION

Recent studies concerning the metabolisms of tricarboxylic acids in microorganisms performed by many workers have revealed an interesting fact that various organisms can effect a C_2 plus C_4 cleavage of citrate, in addition to the formation of α-ketoglutarate by way of the Krebs cycle. The cleavage of tricarboxylic acids into C_2 and C_4 fragments proceeds according to the following reactions:

(1) Citrate→oxalacetate+acetate (citritase)[1~11],
(2) Citrate⇌isocitrate→glyoxylate+succinate (isocitritase)[12~18].

In the previous papers of this series, the authors have demonstrated that the bacteria of *coli-aerogenes* possess an exceedingly high potency of producing α-ketoglutaric acid from varied carbonaceus substances including glucose, pyruvate, lactate and C_4-dicarboxylic acids such as succinate, fumarate, malate and oxalacetate[19~24]. Moreover, it has been found that the washed and treated cells of the bacteria are capable

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of oxidizing the tricarboxylic acids, but little formation of α-ketoglutaric acid is observed, while these dried cells accumulate glyoxylate during the oxidation of citrate in the presence of added trapping agents such as semicarbazide. This indicates that the major oxidation of citrate by the bacteria is accompanied with a cleavage-reaction (glyoxylate plus succinate formation), although a part of oxidation may proceed by way of a isocitrate-α-ketoglutarate-succinate system[24]. Thus, the authors have concluded that the major production of α-ketoglutaric acid from the varied substances, such as glucose, C_3- and C_4- acids, by *coli-aerogenes* does not proceed by way of the conventional tricarboxylic acid cycle, but takes place by way of a certain reaction in which pyruvate and acetate are involved[23,24].

In the preceding paper[25], the authors have also investigated the effects of various antibiotics on the respiration and α-ketoglutaric acid-fermentation in the bacteria, and, the present paper deals with the mechanism of the degradation of citrate caused by the bacteria.

EXPERIMENTAL AND RESULTS

Microorganisms: Three strains of the bacteria, i.e. *Escherichia coli* (strain G–2), *Aerobacter aerogenes* (strain B–2, *coli-aerogenes* (strain B–T), were used throughout the experiments.

Methods: Analytical procedure was performed by the methods mentioned in the previous poper[19~25]. Triphosphopyridine nucleotide (TPN) was obtained from the Tokyo Kasei Co. Ltd.

Degradation of Citrate by Cell-Free Extract of *Coli-aerogenes* Bacteria. In the previous paper[24], it has been ascertained that the dried cells of *A. aerogenes* (strain B–2) grown on citrate or those of *E. coli* (strain G–2) grown on glucose-medium, accumulate glyoxylate in the media as a sort of degradated product of citrate in the presence of externally added trapping agents such as semicarbazide[24]. In the present paper, the authors at first, investigated the degradation of citrate by the cell-free extracts of *coli-aerogenes*.

E. coli was grown on a shaker at 30°C for 2 days in a medium containing 2% glucose, 0.1%$(NH_4)_2HPO_4$, 0.3% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 5 mg% $FeSO_4 \cdot 7H_2O$, 0.2% NaCl and 1% $CaCO_3$. In the case of *A. aerogenes* (B–2), the following medium was employed: 3% Na-citrate, 1% each chrysalis extract and peptone, 0.2% NaCl, 0.3% $(NH_4)_2SO_4$, 0.1% KH_2PO_4 and 0.05% $MgSO_4 \cdot 7H_2O$ (citrate medium). After cultivation, the bacterial cells were harvested with a centrifuge and washed once with distilled wate. The cells were suspended to a level of 20~30 mg of dry weight per ml of distilled water and then disintegrated by treatment with a 10 kc. Raytheon oscillator for 15~25 minutes. The cell debris was removed by a centrifuge, and the cell-free extract was used without dialysis in the experiments.

The reaction mixtures (10~25 ml) containing 1.7mM phosphate buffer (pH: 7.3), 30 μM $MgSO_4$, 200 μM Na-citrate and 5~15 ml cell-free extracts, were dispensed in a Thunberg tube and incubated at 37°C under anaerobic conditions (in vacuo,). The results are shown in Table I, from which it has been observed that glyoxylate and succinate are produced in addition to pyruvate and α-ketoglutarate when the cell-free extracts are anaerobically incubated in the presence of citrate.

Keto-acids observed in the experiments are considered to be produced by way of the following reactions:

$$\text{Citrate} \xrightarrow[\text{citritase}]{\text{splitting reaction}} \text{oxalacetate} + \text{acetate}$$
$$\text{oxalacetate} \rightarrow \text{pyruvate} + CO_2$$
$$\text{Citrate} \xleftarrow{\text{isocitritase}} \text{glyoxylate} + \text{succinate}$$
$$\text{Citrate} \xleftarrow{\text{isocitric dehydrogenase}} \alpha\text{-ketoglutarate} + TPNH_2 + CO_2$$

TABLE I. ANAEROBIC DEGRADATION OF CITRATE BY CELL FREE EXTRACTS OF *Coli-aerogenes*

Bacterial strains	*E. coli* (G–2) grown aerobically on a glucose-medium	*A. aerogenes* (B–2) grown aerobically on a citrate-bouillon medium
Cell-free extracts (ml)	15.0	5.0
(mg Bacterial cells used)	(450)	(130)
Reaction mixtures (ml)	25	10
Time of incubation (hours)	4	2
Pyruvate found (μM)	3.0	2.1
Glyoxylate found (μM)	8.5	30.6
α-Ketoglutarate found (μM)	3.0	Trace
Succinate found (μM)	30.5	38.3

It is of interest to note that *coli-aerogenes* grown aerobically on either glucose or citrate, may possess the ability to degradate citrate to form acetate and oxalacetate, the latter subsequently being decomposed to pyruvate and CO_2.

Effect of Triphosphopyridine Nucleotide on Degradation of Citrate. It has already been demonstrated by Ajl et al. that the cell extracts obtained from the bacteria of genus *Escherichia* such as *E. coli* and *E. freundii* produce α-ketoglutarate from citrate or isocitrate.[8,9,26]

The present authors investigated the influence of addition of TPN on the degradation of citrate by cell-free extracts of *coli-aerogenes*. In order to obtain bacterial cells with a high citritase activity, strain B–T was cultured according to the method of Takahashi et al.[11], who found that when *Bact. succinicum* grown aerobically on citrate-bouillon was again incubated in the presence of glucose and citrate under anaerobic conditions, the activity of citrate-splitting enzyme (citritase) was remarkably increased. In the present investigation, *coli-aerogenes* (B–T strain) was grown on a shaker at 30°C for 20 hours in a medium containing 3% Na-citrate, 1% bouillon, 0.1% KH_2PO_4, 0.3% $(NH_4)_2SO_4$, and 0.05% $MgSO_4 \cdot 7H_2O$ and thereafter incubated at 37°C for 15 hours in the presence of 1% glucose under static conditions (citrate-glucose grown cells). The cells obtained have been observed to degradate citrate to evolve CO_2 under anaerobic conditions. The cell extracts were prepared with a sonic oscillator in the same manner as mentioned above. Incubations were carried out anaerobically at 37°C for 4 hours in either the presence or absence of addition of TPN with the reaction mixture (15 ml) containing 2.5 mM phosphate buffer (pH : 5.7~7.3), 60 μM $MgSO_4$, 100 μM Citrate and 3 ml fresh cell extracts (obtained from 30 mg cells). Table II shows

Table II. Effect of Addition of TPN on Anaerobic Degradation of Citrate by Cell Free Extracts Obtained from Citrate-Glucose-Grown Cells of *Coli-aerogenes* (B–T) Strain

Addition of TPN (γ/ml)	None	30	30
Initial pH	5.7	5.7	7.3
Keto- acids found after 4 hours' incubation			
Pyruvate (μM)	7.2	5.7	5.5
Glyoxylate (μM)	3.8	1.1	Trace
α-Ketoglutarate (μM)	1.2	5.0	5.8

that considerable amounts of glyoxylate and pyruvate in addition to a small amount of α-ketoglutarate are produced during the anaerobic fermentation of citrate in the absence of added TPN, whereas an increasing amount of α-ketoglutarate against decreasing amount of glyoxylate is observed in its presence. Thus, it is evident that the bacterial strain employed here possesses an enzyme isocitric dehydrogenase dependent to triphosphopyridine nucleotide.

Degradation of Citrate by Intact (Washed) Cells of *A. aerogenes*. Table III shows the results of experiments with the intact (washed) cells of *A. aerogenes* grown on citrate medium in the same manner as already mentioned. The reaction mixtures contained 2~5 mM

Table III. Static Incubation of Washed Cells of *A. Aerogenes* Grown on Citrate-Medium

Growth-conditions	*A. aerogenes* (B–2) grown aerobically for 1 day on 3% citrate-1% peptone	*A. aerogenes* (B–2) grown aerobically for 1 day on 3% citrate-1% peptone and thereafter incubated for 15 hours in presence of 1% glucose under static conditions		
Washed cells (mg)	370	390	320	320
Citrate added (μM)	1000.0	1000.0	1000.0	1000.0
Semicarbazide added (mM)	4.5	4.5	3.0	0
Total volume (ml)	50	50	35	35
Time of incubation (hours)	15	10	10	10
Glyoxylate found (μM)	105.0	157.0	154.0	0
α-Ketoglutarate found (μM)	Trace	Trace	Trace	Trace
Acetate found (μM)			Trace	780.0

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phosphate buffer (pH : 7.5～7.8), 50 μM $MgSO_4$ and the amounts of citrate (Na-salt), semicarbazide and washed cells as indicated in Table III, and the reactions were carried out statically at 37°C. From Table III it will be seen that the intact cells of *A. aerogenes* (B–2) grown on citrate excrete a large amount of glyoxylate in the external medium as a degradated product of citrate under conditions where semicarbazide is present, whereas, acetate appears in place of glyoxylate in the external medium where the trapping agent is absent.

On the other hand, the oxidations of varied substrates were investigated with the intact cells of *A. aerogenes*. Incubation mixtures (50 ml) contained 5～10 mM Phosphate buffer (pH : 6.2), 50 μM $MgSO_4$ and the amounts of substrates (Na-salts) and washed cells of *A. aerogenes* grown in the condions as indicated in Table IV; the reactions were carried out at 30°C on the shaker. The results are shown in Table IV, from which it is observed that a remarkable excretion of α-ketoglutarate takes place in the external medium if the intact cells of *A. aerogenes* are aerobically incubated in the presence of pyruvate plus acetate or succinate plus pyravate, while little or no excretion of α-ketoglutarate occurs in the presence of citrate without semicarbazide. These results appear to indicate that the major oxidation of citrate by the intact cells of *A. aerogenes* does not proceed by way of the conventional tricarboxylic acid cycle (citrate → α-ketoglutarate → succinate), but occurs by way of a splitting reaction in which isocitritase is concerned. It should now be noted that the oxidations of C_3 and C_4-acids give rise to a large amount of α-ketoglutaric acid, that is, the oxidations of these substrates proceed toward the formation of α-ketoglutarate. Accordingly, it has strongly been suggested that the mode of the major oxidation of C_3- and C_4-acids including pyruvate, lactate, succinate, fumarate, malate and oxalacetate may be of a different type from that of tricarboxylic acids.

Degradation of Citrate by Dry- and Ground Dry-cells of *Coli-aerogenes*. The results mentioned above were again tested with both dry- and ground dry-cells of *coli-aerogenes*. The cells of *E. coli* (G–2 strain) grown on a glucose-medium and those of *A. aerogenes* (B–2 strain) and *coli-aerogenes* (B–T strain) grown on a citrate-medium were dried by an electric fan at room temperature for 5 hours. Ground cells of the bacteria were prepared by the methods mentioned in the previous paper[24]. Both anaerobic and aerobic degradations of citrate by these treated cells were carried out at 30°C in the presence and absence of externally added semicarbazide.

In both cases, the oxidation of pyruvate plus acetate by the treated cells was tested for the sake of comparison. The results are shown in Tables V and VI, from which it has been ascertained that a remarkable accumulation of glyoxylate takes place in addition to the formation of pyruvate when the dry- and ground dry-cells (250～800 mg) of *coli-aerogenes* are incubated with the

TABLE IV. OXIDATION OF VARIED SUBSTRATES BY WASHED CELLS OF *A. aerogenes* GROWN ON CITRATE-MEDIUM

Growth-conditions	*A. aerogenes* (B–2) grown aerobically for 2 days on 3% citrate-1% peptone		*A. aerogenes* (B–2) grown aerobically for 2 days on 3% citrate-1% bouillon -0.5% yeast extract		*A. aerogenes* (B–2) grown aerobically for 1 day on 3% citrate-1% bouillon and thereafter incubated statically for 15 hours in presence of 1% glucose	
Substrates against which washed cells were tested (μM)	Citrate 1500.0	Pyruvate 3000.0 plus acetate 2000.0	Citrate 1500.0	Pyruvate 2000.0 plus acetate 1000.0	Pyruvate 2000.0 plus succinate 1000.0	Citrate 1500.0
Bacterial cells (mg)	100	100	270	270	320	320
Semicarbazide added (mM/50 ml)	0	0	4.5	0	0	0
Time of incubation (hours)	13	13	6	6	5	5
Consumption of substrates (%)	100	100		100	100	100
Pyruvate found (μM)	0	0	8.1	0	0	0
Glyoxylate found (μM)	0	0	5.1	0	0	0
α-Ketoglutarate found (μM)	Trace	510.5	9.9	202.5	555.6	50.3

Table V. Aerobic Degradation of Pyruvate Plus Acetate and Citrate by Dry— and Ground Dry— Cells of *E. coli* (G–2) Grown Aerobically on Glucose-Ammonium Medium

Substrates added (μM)	Pyruvate plus acetate 1500.0	1000.0	Citrate 500.0	Citrate 500.0	Citrate 500.0
Bacterial cells of *E. coli* (mg)	Dry-cells 300		Dry-cells 300	Dry-cells 300	Ground Dry-cells 800
Semicarbazide added (mM)	0		4.5	0	4.5
Reaction mixtures (initial pH)	6.3		7.2	7.2	7.4
(ml)	50		50	50	50
Time of incubation (hours)	5		5	5	5
Glyoxylate found (μM)	0		14.8	0	35.5
Pyruvate found (μM)	200.0 (consumed 1300.0)		4.4	0	25.4
α-Ketoglutarate found (μM)	90.6		3.5	Trace	Trace

Table VI. Degradation of Citrate by Ground Dry—Cells of *Coli-aerogenes*

Growthconditions	*Coli-aerogenes* (B–T) grown aerobically for 1 day on 3% citrate-2% peptone medium		*Coli-aerogenes* (B–T) grown aerobically for 1 day on 3% citrate-1% peptone and thereafter incubated statically for 15 hours in presence of glucose		*A. aerogenes* (B–2) grown aerobically for 1 day on 3% citrate -0.5% peptone		
Dried cells (mg)	320	320	250	250	430	430	430
Citrate added (μM)	500.0	500.0	500.0	500.0	1000.0	1000.0	1000.0
Semicarbazide added (mM)	2.5	2.5	2.2	2.2	3.3	3.3	0
Reaction mixtures (initial pH)	7.5	7.5	7.5	7.5	7.3	7.3	7.3
Reaction mixtures (ml)	30	30	25	25	45	45	45
Time of incubation (hours)	5 Shaking	5 Static	6 Shaking	6 Static	5 Shaking	5 Static	5 Static
Pyruvate found (μM)	20.5	25.3	65.0	68.5	Trace	Trace	Trace
Glyoxylate found (μM)	76.5	102.0	68.0	72.0	237.0	216.5	Trace
α-Ketoglutarate found (μM)	Trace	Trace	Trace	Trace	Trace	Trace	Trace

media (25~50 ml) containing 2.5~5.0 mM phosphate buffer (pH: 6.3~7.5), 50~100 μM $MgSO_4$, 2.2~4.5mM semicarbazide and 500~1000 μM citrate under either aerobic or anaerobic conditions. In the case of the treated cells of strain B–T, the preparations have been ascertained to contain citritase (citrate → oxalacetate + acetate), and therefore it has strongly been suggested that pyruvate detected during the degradation of citrate by the preparations may be derived from oxalacetate. Tables V and VI also show that the degradation of citrate by the treated cells results in yielding only a very small amount of α-ketoglutarate even under conditions in which a trapping agent such as semicarbazide is present. Thus, it has been concluded that a considerable portion of citrate is metabolized to glyoxylate and succinate or to oxalacetate and acetate by the action of isocitritase and citritase respectively, rather than to succinate via the isocitric-α-ketoglutaric dehydrogenase system. It is already known that the following reactions proceed at a very slow rate: the oxidation of citrate by *coli-aerogenes* grown on a glucose-medium, and also the oxidation of α-ketoglutarate by the bacteria grown on either glucose- or citrate-medium.

Occurrence of Isocitritase in *Coli-aerogenes* Grown under Various Conditions. It has been reported by Smith and Gunsalus that when *E. coli* is grown with glucose as the source of energy, the bacterial cells formed are devoid of isocitritase, whereas, activity of the enzyme is high when the organism is grown aerobically with a medium containing acetic acid as a source of energy[17].

However, from the experiments described in the pre-

ceding and in the present papers, it wll be clear that *E. coli* grown aerobically on a glucose-medium actually possesses isocitritase, and it thus appears that the low activity of isocitritase corresponds to a low potency of metabolizing (oxidizing) citrate under these conditions.

Subsequently, the authors have examined the activity of isocitritase in *coli-aerogenes* obtained under various growth-conditions. The results are shown in Table VII, in which experiments were carried out with various preparations of the bacteria such as dry- and ground-cells, and cell-free extracts in the same manner as already mentioned. It can be seen that isocitritase exists in all of the bacterial cells examined, the growth-media used being as follows: bouillon-, peptone-, glucose-, acetate plus bouillon-, succinate plus bouillon-, and citrate plus bouillon (peptone)-media. It should be noted that *coli-aerogenes* grown anaerobically on a citrate plus bouillon-medium possesses considerable activity of isocitritase.

SUMMARY

1) Glyoxylate and succinate were formed in addition to pyruvate and α-ketoglutarate, when the cell free extracts of *coli-aerogenes* grown on either glucose or citrate were incubated in the presence of citrate.

TABLE VII. OCCURRENCE OF ISOCITRITASE IN *Coli-aerogenes* BACTERIA

Bacterial strains (Strain No.)	Growth-media	Enzyme preparations	Semicarbazide added (μM/ml)	Glyoxylate formed by 100 mg cells per hour at 30–37°C (μM)
E. coli (G-2)	2% Glucose-NH_3-N aerobically	Dried cells	50~100	0.6~3.0
		Cell-free extracts	0	0.3
A. aerogenes (B-2)	3% Citrate-0.5% peptone aerobically	Cell-free extracts	0	4.0
		Dried cells	75	10.0
A. aerogenes (B-2)	1% Peptone-1% meat exts. aerobically	Dried cells	75	5.0
Coli-aerogenes (B-T)	1% Peptone-1% meat exts. aerobically	Sonically disintegrated cells	0	5.7
Coli-aerogenes (B-T)	2% Peptone aerobically	Dried cells	75	3.0
Coli-aerogenes (B-T)	2% Succinate-1% peptone aerobically	Cell-free extracts	0	2.2
Coli-aerogenes (B-T)	1% Acetate-bouillon aerobically	Cell-free extracts	0	10.0
Coli-aerogenes (B-T)	3% Citrate-bouillon aerobically	Cell-free extracts	0	4.5
Coli-aerogenes (B-T)	3% Citrate-bouillon statically	Cell-free extracts	0	2.0

2) The fresh cell extracts of *coli-aerogenes* revealed a very poor potency of producing α-ketoglutarate with citrate, whereas, the addition of coenzyme II (TPN) to the reaction mixtures resulted in an increase in formation of α-ketoglutarate from citrate.

3) It was found with *coli-aerogenes* that isocitritase existed in the various cells obtained from the following growth- media: bouillon-, peptone-, glucose-, acetate plus bouillon- succinate plus bouillon- and citrate plus bouillon (Peptone)- media.

4) Isocitritase was demonstrable, even when *coli-aerogenes* was anaerobically grown on a citrate-bouillon medium.

5) A large amount of glyoxylate was excreted in the external media, when not only the dried and ground cells of *coli-aerogenes*, but the intact (washed) cells of *A. aerogenes* were also incubated in the presence of citrate and a trapping agent such as semicarbazide.

6) Isocitric dehydrogenase existed in the cells of *coli-aerogenes*.

7) From the results obtained in the previous and present papers, it was strongly suggested that the intracellular concentration of coenzyme II of *coli-aerogenes* might be relatively low, so that coenzyme II-dependent isocitric dehydrogenase could not be operative at such a high rate sufficient to effect the complete removal of citrate.

8) It was indicated that the major degradation of C_6-tricarboxylic acids by *coli-aerogenes* took place by way of splitting reactions rather than depending on the isocitric-α-ketoglutaric dehydrogenase system.

9) With both C_3- and C_4-acids, α-ketoglutarate was again ascertained to be obtainable in a high yield.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 6, p. 482~489, 1959]

Microbiological Studies of *Coli-aerogenes* Bacteria*
Part VIII. The Occurrence of Isocitritase and Citritase

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Received February 3, 1959

The bacteria of *coli-aerogenes* were found to contain two enzymes in relation to the splitting reaction of citrate i.e., isocitritase and citritase when the organisms were grown on citrate. The cell extracts of two strains were fractionated with ammonium sulfate for isocitritase and citritase. Consequently, the action of isocitritase was remarkably inhibited in the presence of a large amount of succinate added, whereas, in citritase this was not observed at all. Antibiotics such as dihydrostreptomycin, chloramphenicol and tetracycline were never observed to produce any inhibiting effect on both citrate-splitting enzymes. Thus, the mechanism of degradation of citrate by the bacteria was suggested.

INTRODUCTION

Recent studies on the dissimilation of the C_6-tricarboxylic acids in microorganisms have demonstrated that the following cleavage-reactions exist in addition to the stepwise degradation of C_6-acids by way of the Krebs cycle:

(I) Citrate $\xrightarrow{\text{citritase}}$ oxalacetate + acetate[1~16]

(II) Citrate $\rightleftarrows$ isocitrate $\xrightarrow{\text{isocitritase}}$ glyoxylate + succinate[17~23,28,30]

* This paper was read before the Meeting of the Japanese Biochemical Society, held at Kyoto University (November, 1958).

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In the case of genus *Aerobacter*, it has been reported by several workers that the bacterial cell grown on citrate under aerobic conditions, can primarily metabolize citrate by the conventional tricarboxylic acid cycle[31,32], while the cell grown anaerobically on citrate can degradate citrate directly to acetate and oxalacetate[1~3,8]. On the other hand, it has been indicated by Smith and Gunsalus that *Escherichia coli* grown aerobically with acetate as a source of energy, contains isocitritase, whereas, the cell grown on glucose under either aerobic or anaerobic conditions, is devoid of isocitritase[21,23].

They have suggested that in *E. coli*, citritase and isocitritase are operative anaerobically and

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aerobically, respectively.

In the course of the investigations on the oxidative fermentation of *coli-aerogenes*, the authors have found that the bacteria possess an exceedingly high ability to produce α-ketoglutaric acid as a major oxidation-product of the varied carbonaceus substances including glucose, lactic, pyruvic and C_4-dicarboxylic acids[24~28]. Further investigations have demonstrated that these organisms reveal an exceedingly low potency to induce α-ketoglutaric acid-fermentation when C_6-tricarboxylic acids are employed as the sole source of carbon[28,30]. In this case, C_6-tricarboxylic acids have been ascertained to be degradated into glyoxylate and succinate: that is to say, a cleavage-reaction of C_6-acids into C_2 and C_4 units takes place at a much higher rate than that of the stepwise degradation of C_6-acids by way of the Krebs cycle[28,30].

In the present investigations, the authors' attention has been directed to another cleavage-reaction by which oxalacetate and acetate are anaerobically formed from citrate, and also to elucidate the relationship between these two enzymes splitting C_6-acids.

EXPERIMENTAL AND RESULTS

Experimental procedures were performed by methods mentioned in the previous papers of this series. Glutathione was generously supplied by Dr. Y. Kuroiwa of Kirin Research Institute.

Fractionation of Cell Free Extracts In the present paper, experiments were at first, carried out with the following two strains of *coli-aerogenes*: strain B-T with high abilities to produce both citritase and isocitritase, strain B-2 with an exceedingly high potency of isocitritase but with a low potency of citritase. These organisms were grown on a shaker at 30°C for 20 hours on a medium (pH 7.2) containing 3% Na-citrate, 1% of each peptone and chrysalis extracts, 0.5% yeast extracts, 0.1% KH_2PO_4, 0.3% $(NH_4)_2SO_4$, 0.1% $(NH_4)_2HPO_4$, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.2% NaCl, and thereafter glucose (the final concentration 1 g/dl) was added to the cultured media and, subsequently incubations were continued statically at 37°C for a period of 15 hours (citrate-glucose grown cells). After cultivation, the bacterial cells were harvested with a centrifuge and washed once with distilled water. In the case of strain B-T, it was ascertained that the cell possessed not only high ability of degradating citrate to form acetate, succinate and carbon dioxide under anaerobic conditions, but also that of decarboxylating oxalacetate to pyruvate and carbon dioxide. The cells were suspended to a level of 15~35 mg of dry weight per ml of distilled water and then disintegrated by treatment with a 10 kc. Raytheon oscillator for 15~30 minutes. The cell debris was removed by a centrifuge and the cell-free extracts obtained were fractionated for isocitritase and citritase. Solid ammonium sulfate was added to the cell-free extracts so as to give the various saturations as indicated in Tables I, II, and III, and the precipitates formed were collected by a centrifuge and dissolved in 0.1 M phosphate buffer, pH 7.5. The various ammonium sulfate saturation fractions were dialysed against 0.05 M phosphate buffer (pH 7.5) at 5°C for 20~40 hours. The results of experiments with ammonium sulfate ($AmSO_4$) fractions are shown in Tables I (a), (b), II and III. Table I (a): the reaction mixture (10 ml) contained 2.5 mM phosphate buffer (pH 5.7~8.3), 100 μM Na-citrate, 2ml $AmSO_4$ 0~0.3 fraction obtained from 60 mg cells of B-T strain and the stated amounts of $MgSO_4$ and Na-arsenite; 120 minutes' incubation anaerobically at 37°C. Table I (b): the reaction mixture (10 ml) contained 1.0 mM phosphate buffer (pH 7.2), 100 μM Na-citrate, 10 μM glutathione, the stated amounts of $MgSO_4$ and 3 ml

TABLE I. OCCURRENCE OF ISOCITRITASE AND CITRITASE IN B-T STRAIN OF *Coli-aerogenes*

Enzymes	Experiment I(a)				Experiment I(b)		
	$AmSO_4$ 0~0.3 fraction				$AmSO_4$ 0~0.3 fraction	$AmSO_4$ 0.3~0.6 fraction	
Initial pH	5.7	5.7	5.7	8.3	7.2	7.2	7.2
Arsenite added (M/l)	0	4×10^{-3}	0	0	0	0	0
$MgSO_4$ added (μM)	40	40	0	40	40	0	40
	120 minutes' incubation				90 minutes' incubation		
Pyruvate found (μM)	7.5	7.7	0	4.0	1.3	0	2.5
Glyoxylate found (μM)	Trace	Trace	0	Trace	Trace	0	4.0

TABLE II. FRACTIONATION OF CELL EXTRACTS OF B-T Strain OF *Coli-aerogenes*

$AmSO_4$ fraction	0~0.3 saturation		0.3~0.4 saturation		0.4~0.6 saturation	
Initial pH	5.7	7.3	5.7	7.3	5.7	7.3
180 minutes' incubation						
Pyruvate found (μM)	3.6	2.7	4.3	2.9	0.5	Trace
Glyoxylate found (μM)	Trace	Trace	0.7	0.9	2.2	3.0
α-Ketoglutarate found (μM)	0	0	0	0	0	0
Succinate found (μM)					2.5	3.7

TABLE III. FRACTIONATION OF CELL EXTRACTS OF B-2 Strain OF *A. aerogenes*

	Experiment (A)			Experiment (B)		
$AmSO_4$ fraction	0~0.4 saturation	0.4~0.6 saturation		0.4~0.6 saturation		
Substrates added (μM)	Citrate	Citrate	Citrate	Citrate	cis-Aconitate	iso-Citrate
	50.0	50.0	50.0	60.0	60.0	60.0
Glutathione added (μM)	12	12	0	16	16	16
$MgSO_4$ added (μM)	15	15	0	15	15	15
180 minutes' incubation						
Glyoxylate found (μM)	Trace	7.5	0	5.1	2.7	1.9

$AmSO_4$ fractions (0~0.3, 0.3~0.6) obtained from 100 mg cells of strain B-T; 90 minutes' incubation anaerobically at 37°C. Table II: the reaction mixture (10 ml) contained 2.0 mM phosphate buffer (pH 5.7~7.3), 60 μM $MgSO_4$, 100 μM Na-citrate and 3 ml $AmSO_4$ fractions (0~0.3, 0.3~0.4, 0.4~0.6) obtained from 55 mg cells of strain B-T; 180 minutes' incubation anaerobically at 37°C. Table III: the reaction mixture (7 ml) contained 0.5 mM phosphate buffer (pH 7.4), stated amounts of $MgSO_4$, glutathione and substrates (Na-salts) and 2 ml $AmSO_4$ fractions (0~0.4, 0.4~0.6) obtained from 60 mg cells of strain B-2; 180 minutes' incubation anaerobically at 37°C.

From the results shown in Table I, it has been found that ammonium sulfate 0.3 saturation-fraction prepared from strain B-T of *coli-aerogenes* reveals an activity of citritase but little or no activity of isocitritase, while ammonium sulfate 0.3~0.6 saturation-fraction reveals high activities of both citritase and isocitritase. Table II also shows that a 0.3~0.4 ammonium sulfate saturation-fraction possesses activities of both citritase and isocitritase, whereas, the 0.4~0.6 fraction possesses high activity of isocitritase against the extremely low activity of citritase. On the other hand, in the case of another strain (B-2) of *A. aerogenes* which has a very much high activity of isocitritase but reveals an extremely low activity of citritase, it may be indicated from Table III that when the 0~0.4 ammonium sulfate fraction is used, little or no activity of isocitritase is observed, while the 0.4~0.6 fraction reveals a high activity of isocitritase. Thus, it appears that both the aconitase and isocitritase may be precipitated at a lower concentration of added ammonium sulfate in strain B-T with a high activity of citritase than in strain B-2 with a low activity of the enzyme, and that also a part of isocitritase in strain B-T may be precipitated at approximately the same concentration of ammonium sulfate as required in the citritase, although a considerable portion of isocitritase of the strain B-T tends to be precipitated at a higher concentration of ammonium sulfate than that in citritase of the strain. These facts may suggest that citritase which is an adaptive enzyme is formed by an enzymic modification of isocitritase.

In the previous paper[30], it has already been demonstrated by the authors that the formation of isocitritase in *coli-aerogenes* takes place under the various growth-conditions, and this may indicate that isocitritase is a constitutive enzyme. Citrate has been observed to serve as a substrate with various ammonium sulfate fractions The properties of aconitase of *coli-aerogenes* are now under investigation. Magnesium ion is ascertained to be indispensable for the activities of isocitritase and citritase (see Table I).

TABLE IV. ANAEROBIC DEGRADATION OF CITRATE BY CELL EXTRACTS OF *Coli-aerogenes*

Bacterial cells	B–T strain of *coli-aerogenes* grown on citrate		B–2 strain of *A. aerogenes* grown on citrate	G–2 strain of *E. coli* grown on glucose
Cell extracts added (ml)	5	5	1	4
(mg cells used)	(150)	(150)	(30)	(110)
Reaction mixtures (ml)	10	10	8	10
Time of incubation (hours)	4	4	3	4
Pyruvate found (μM)	9.0	13.2	0.8	2.3
Glyoxylate found (μM)	7.5	3.5	10.5	10.6
α-Ketoglutarate found (μM)	3.5	2.1	Trace	1.5

Anaerobic Degradation of Citrate by Cell-Free Extracts of *Coli-aerogenes*. Table IV shows the results of experiments conducted with the cell-free extracts of three kinds of the bacterial strains of *coli-aerogenes*. Incubations were carried out anaerobically at 37°C with the media (8~10 ml) containing 1 mM phosphate buffer (pH 7.5), 40 μM $MgSO_4$, 100 μM Na-citrate and the amounts of the sonically treated cell extracts of the bacteria as indicated in Table IV. From the Table it can be seen that although the cell extracts produce three kinds of keto-acids, i.e., glyoxylate, pyruvate and α-ketoglutarate from citrate, the proportion of glyoxylate to pyruvate is greatly dependent upon the growth-conditions and also vary from strain to a strain. In all of the cases examined, α-ketoglutarate was obtained in only an extremely low yield. The anaerobic degradation of citrate to succinate by the bacteria of *coli-aerogenes* has been frequently indicated to proceed by way of a coupling reaction of the following type:

$$\text{Citrate} \xrightarrow{\text{citritase}} \text{acetate} + \text{oxalacetate} \quad (1)$$

$$\text{Citrate} \xrightarrow{\text{TCA cycle}} \text{succinate} + 4\text{H} + 2\,CO_2 \quad (2)$$

$$\text{Oxalacetate} + 4\text{H} \longrightarrow \text{succinate} \quad (3)$$

$$\text{Sum: 2 Citrate} \longrightarrow 2\ \text{succinate} + \text{acetate} + 2\,CO_2 \quad (4)$$

From the results mentioned in previous and in the present papers, it appears that a considerable portion of citrate may be metabolized to acetate, carbon dioxide and succinate under anaerobic conditions, according to the coupling reaction of this type. On the other hand, the anaerobic degradation of citrate also appears to involve a coupling reaction of another type. It has been suggested by the authors that some portion of citrate may be metabolized according to the following mechanism:

$$\text{Citrate} \xrightarrow{\text{citritase}} \text{acetase} + \text{oxalacetate} \quad (5)$$

$$\text{Citrate} \xrightarrow{\text{isocitritase}} \text{glyoxylate} + \text{succinate} \quad (6)$$

$$\text{Oxalacetate} \longrightarrow \text{acetate} + 2\text{H} + 2CO_2 \quad (7)$$

$$\text{Acetate} + \text{glyoxylate} + 2\text{H} \longrightarrow \text{succinate} \quad (8)$$

$$\text{Sum: 2 Citrate} \longrightarrow 2\ \text{succinate} + \text{acetate} + 2CO_2 \quad (9)$$

In this case, isocitritase, decarboxylating system of oxalacetate to acetate via pyruvate, and C_4-dicarboxylic acid-synthesizing system in addition to citritase may be involved in the degradation procedure of citrate. It has already been found by Wong and Ajl that *E. coli* possesses malate synthetase which effects the formation of malate from glyoxylate and acetyl-coenzyme A[35].

Influence of Addition of Organic Acids upon Activities of Citritase and Isocitritase.

It has been reported by Takahashi[13~15] that the formation of pyruvate occurs when the cell-free extracts of *Bact. succinicum* trained to citrate under anaerobic conditions are anaerobically incubated in the presence of citrate, and that the addition of a large amount of succinate or arsenite to the reaction mixture results in an increase in the formation of pyruvate. It is of interest to ascertain whether or not the anaerobic degradation of citrate by strain B–T with both citritase and isocitritase or by strain B–2 with extremely high activity of isocitritase would be influenced by the presence of added succinate. The experiments carried out with strain B–T are shown in Table V, in which the reaction mixture (15 ml) contained 2 mM phosphate buffer (pH 6.2~7.4), 44 μM $MgSO_4$, 100 μM citrate and 3 ml cell extracts prepared from 54 mg cells of strain B–T and the indicated amounts of Na-succinate and Na-arsenite;

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TABLE V. INFLUENCE OF ADDITION OF SUCCINATE UPON ANAEROBIC DEGRADATION OF CITRATE BY CELL EXTRACTS OF *Coli-aerogenes* (B–T Strain) GROWN ON CITRATE

Exp. No.	I	II	III	IV	V
Initial pH	6.2	6.2	6.2	7.4	7.4
Conc. of additions (M/l)	None	Na-succinate 3.3×10^{-2}	Na-succinate plus 3.3×10^{-2} Na-arsenite 2.7×10^{-3}	None	Na-succinate 3.3×10^{-2}
Keto acids found after 3 hours' incubation					
Pyruvate (μM)	9.0	11.1	12.2	7.5	9.7
Glyoxylate (μM)	3.9	0	0	3.3	0

TABLE VI. INFLUENCE OF ADDITION OF SUCCINATE UPON ISOCITRITASE OF *Coli-aerogenes* (B–T Strain) GROWN ON CITRATE

Addition of succinate (M/l)	None	3.1×10^{-3}	6.3×10^{-3}	1.3×10^{-2}	1.8×10^{-2}	3.1×10^{-2}
Glyoxylate found after 3 hours' incubation (μM)	4.7	4.0	2.0	Trace	0	0

TABLE VII. INFLUENCE OF ADDITIONS OF VARIOUS ACIDS UPON ISOCITRITASE OF *Coli-aerogenes* (B–T Strain) GROWN ON CITRATE

(A) With $AmSO_4$ 0~0.5 fraction, reaction mixture 20 ml, 4 hours' incubation

Additions (μM/20 ml)	None	Acetate 1000	Succinate 500
Glyoxylate found (μM)	13.6	14.4	2.5

(B) With $AmSO_4$ 0.3~0.6 fraction, reaction mixture 15 ml, 4 hours' incubation

Additions (μM/15 ml)	None	Succinate 500	Fumarate 500	DL-Malate 500
Glyoxylate found (μM)	24.1	Trace	15.7	24.7

3 hours' incubation anaerobically at 37°C. It has been found that the presence of added-succinate does not produce any inhibiting effect upon the activity of citritase but brings about a complete inhibition of isocitritase. Table VI shows the effect of increasing the concentration of succinate on the action of isocitritase, from which it will be seen that complete inhibition of succinate occurs upon isocitritase when a reaction mixture (15 ml) containing 2 mM phosphate buffer (pH 7.5), 60 μM $MgSO_4$, 5 μM glutathione, 100 μM Na-citrate and 2 ml ammonium sulfate 0.3~0.6 fraction (corresponding to 45 mg cells) was anaerobically incubated at 37°C for 3 hours in the presence of a large amount of succinate so as to give a final concentration of 1.3×10^{-2} M/l. On the other hand, Table VII shows the effects of additions of various organic acids, and in this case, incubations were carried out anaerobicaly at 37°C for 4 hours with the media (15~20 ml) containing 1 mM phosphate buffer (pH 7.5) 60 μM $MgSO_4$, 10~20 μM glutathione, 150 μM Na-citrate, 3~4 ml (corresponding to 80 mg cells) ammonium sulfate fraction (0~0.5, 0.3~0.6) and the amounts of various organic acids (Na-salts) as indicated. It will be seen that the action of isocitritase is not inhibited by the presence of added acetate and malate.

Effects of Antibiotics and Other Inhibitors. In the previous paper of this series, the authors have studied the actions of various antibiotics on the respiration and α-ketoglutaric acid-fermentation in *E. coli*[29]. It has been found with both the growing culture and the bacterial cells such as washed or dried cells grown on glucose that dihydrostreptomycin not only depresses the bacterial accumulation of α-ketoglutarate from a variety of carbonaceus substances including glucose, acetic, pyruvic, lactic and C_4-dicarboxylic acids, but also stimulates the oxidative removal of α-ketoglutarate by the organism. Investigations on the action of chloramphenicol have brought out another fact: that chloramphenicol markedly inhibits α-ketoglutarate-synthesizing reaction without causing considerable inhibition of oxidative removal of a variety of carbonaceus substances men-

Table VIII. Effect of Various Antibiotics on Isocitritase

(A) With tetracycline				
Addition of tetracycline (γ/9 ml)	None	1000	None	1000
Initial pH	6.6	6.6	7.4	7.4
Glyoxylate found after 2 hours' incubation (μM)	3.6	3.3	4.4	4.5

(B) With dihydrostreptomycin and chloramphenicol			
Additon of antibiotics (γ/8 ml)	None	Dihydrostreptomycin 1000	Chloramphenicol 1000
Initial pH	7.4	7.4	7.4
Glyoxylate found after 2 hours' incubation (μM)	11.4	11.3	11.4

Table IX. Influence of Various Antibiotics on Citritate of B–T Strain

Antibiotics added (γ/ml)	None	Dihydrostreptomycin 190	Chloramphenicol 190	Tetracycline 190
Pyruvate found after 4 hours' incubation (μM)	10.3	11.3	12.5	12.4

tioned above.

This indicates that the organism possesses both the oxidative pathway sensitive to the drugs and the non-sensitive to them. Tetracycline and the related compounds have been observed to remarkably inhibit the consumption of substrates by the organism.

On the other hand, a number of the studies which have been carried out on the action of antibiotics have demonstrated that the respiration-systems in various bacteria are often inhibited by the presence of the drugs. It has, however, been reported by some workers that streptomycin and chloramphenicol have no inhibiting effect on the formation of citrate from pyruvate (active acetate) and oxalacetate by the bacteria such as *E. coli*[33] and *Pseudomonas fluorescens*[34]. The present paper deals with the actions of dihydrostreptomycin, chloramphenicol and tetracycline upon isocitritase and citritase of the bacteria of *coli-aerogenes*.

Experiments were carried out with the cell-free extracts of the bacteria prepared in the same manner as already mentioned. Experiments on the effect of antibiotics upon isocitritase of strain B–T are shown in Table VIII, in which incubations were anaerobically carried out at 37°C with the reaction mixtures (8~9 ml containing 0.5 mM phosphate buffer (pH 6.6~7.4), 20 μM $MgSO_4$, 50 μM Na-citrate and 2 ml cell extracts obtained from 50 mg cells and the antibiotics indicated. In the case of the experiments on the effect of antibiotics upon citritase of strain B–T (Table IX), the reaction mixture (15 ml) contained 2.5 mM phosphate buffer (pH 5.7), 60 μM $MgSO_4$, 100 μM Na-citrate, 3 ml cell extracts obtained from 30 mg cells, 470 μM Na-succinate and indicated amounts of the antibiotics. Succinate was added to the reaction mixture to inhibit the action of isocitritase present in the cell extracts. From the results shown in Tables VIII and IX, it can be seen that both citritase and isocitritase of strain B–T are not inhibited by the presence of dihydrostreptomycin, chloramphenicol and tetracycline. Table X also shows

Table X. Anaerobic Degradation of Citrate by Cell-Free Extracts of G–2 Strain of *E. coli* Grown Aerobically on Glucose Medium

Addition of tetracycline (γ/ml)	None	200
Fermentation products after 4 hours' incubation		
Pyruvate found (μM)	4.5	5.0
Glyoxylate found (μM)	7.5	7.3
α-Ketoglutarate found (μM)	2.0	2.2
Succinate found (μM)	29.5	28.1

that tetracycline does not inhibit the static degradation of citrate by cell extracts of *E. coli* grown aerobically on a glucose-medium: incubation was statically carried out at 37°C on a medium (25 ml) containing 1.7 mM phosphate buffer (pH 7.3), 30 μM $MgSO_4$, 200 μM Na-citrate, 15 ml cell extracts obtained from 450 mg cells and the stated amount of tetracycline.

Subsequently, the effects of other inhibitors upon the action of isocitritase were tested as follows: the reaction mixtures (25 ml) contained 2.5 mM phosphate buffer (pH 7.5), 30 μM $MgSO_4$, 250 μM Na-citrate, 10 ml cell extracts obtained from 220 mg cells of strain B–T and

TABLE XI. INFLUENCE OF INHIBITORS ON FORMATION OF GLYOXYLATE AND SUCCINATE FROM CITRATE BY CELL EXTRACTS OF B–T STRAIN OF *Coli-aerogenes*

Addition of inhibitors (M/l)	None	NaN_3 8×10^{-3}	$\alpha\alpha'$-Dipyridyl 2×10^{-3}
4 hours' incubation			
Glyoxylate found (μM)	21.6	21.5	21.3
Succinate found (μM)	38.2	34.6	35.5

the inhibitors as indicated in Table XI; incubations were carried out anaerobically at 37°C. From the results shown in Table XI, it can be seen that isocitritase is not inhibited by the presence of NaN_3 and $\alpha\alpha'$-dipyridyl. The following inhibitors also were found to have no effect on the formation of glyoxylate by the cell extracts of strain B–2 of *A. aerogenes*: *o*-phenanthroline (2×10^{-3} M/l), NaF (5×10^{-2} M/l) and 2 : 4 dinitrophenol (1×10^{-3} M/l).

Oxidations of Various Substrates by Washed Cells of Strain B–T. In the previous papers[28~30], the authors, employing a cell of strain B–2 of *A. aerogenes* with a high activity of isocitritase, have investigated oxidations of various substrates such as C_3-monocarboxylic, C_4-dicarboxylic and C_6-tricarboxylic acids, and it has been found that a remarkable excretion of α-ketoglutaric acid occurs in the external medium when the bacterial cell is aerobically incubated with C_3- and C_4- acids, whereas, little or no excretion of α-ketoglutaric acid takes place in the presence of C_6-tricarboxlic acids without a trapping agent such a semicarbazide. In the present paper, the oxidations of pyruvic, succinic and citric acids have again been tested with the washed cells of strain B–T of *coli-aerogenes* grown under the various conditions. It is to be noted that B–T strain reveals an ability of producing a large amount of citritase when the strain is grown under anaerobic conditions in which citrate is present. Incubations were aerobically carried out at 30°C for 10~14 hours with the media (40~50 ml) containing 10 mM phosphate buffer (pH 5.7), 50 μM $MgSO_4$, the amounts of various substrates (Na-salts) and the washed cells of strain B–T grown under various conditions as shown in Table XII. The results are shown in Table XII, frrm which it is again observed that not only the cells of B–T strain with a very much low activity of citritase but those with an exceedingly high activity are also capable of excreting a large amount of α-ketoglutaric acid in the external media when they are incubated aerobically with C_3- and C_4-acids such as pyruvic and succinic acids, while, there is little or no excretion of α-ketoglutaric acid, if citric acid is present as a sole source of carbon.

SUMMARY

1) It was found with *coli-aerogenes* that two kinds of citrate-splitting enzymes, i.e. citritase and isocitritase, were formed when the organisms were grown on citrate.

2) The mode of degradation of citrate was suggested to be as follows:

$$[\mathrm{I}]\quad \mathrm{Citrate} \xrightarrow{\text{citritase}} \text{acetate} + \text{oxalacetate}$$

$$\mathrm{Citrate} \xrightarrow{\text{TCA cycle}} \text{succinate} + 4\mathrm{H} + 2\mathrm{CO_2}$$

$$\text{Oxalacetate} + 4\mathrm{H} \longrightarrow \text{succinate}$$

$$\text{Sum: } 2\ \text{Citrate} \longrightarrow 2\ \text{succinate} + \text{acetate} + 2\mathrm{CO_2}$$

TABLE XII. OXIDATIVE FERMENTATION IN B–T STRAIN OF *Coli-aerogenes*

Growth-conditions	B–T strain grown aerobically on 1% peptone – 2% Na-succinate		B–T strain grown aerobically on 1% bouillon – 2% glucose	B–T strain with high activity of citritase, grown on citrate – glucose	
Washed cells (mg as dry-weight)	120	120	150	100	100
Substrates added (μM)	Pyruvate 4000.0 plus acetate 2000.0	Succinate 2000.0 plus acetate 2000.0	Pyruvate 4000.0 plus acetate 2000.0	Pyruvate 3000.0	Citrate 1500.0
Total volume (ml)	50	50	50	40	40
Time of incubation (hours)	12	12	14	10	10
Consumption of substrates (%)	100	100	100	100	100
α-Ketoglutarate found (μM)	750.5	1000.5	1020.3	185.5	Trace

[II] Citrate $\xrightarrow{\text{citritase}}$ acetate + oxalacetate

Citrate $\xrightarrow{\text{isocitritase}}$ glyoxylate + succinate

Oxalacetate $\longrightarrow$ acetate + 2H + $2CO_2$

Acetate + glyoxylate + 2H $\longrightarrow$ succinate

Sum: 2 Citrate $\longrightarrow$ 2 succinate + acetate + $2CO_2$

3) The cell free extracts of both strains B–T and B–2 of *coli-aerogenes* were fractionated for isocitritase and citritase.

4) The yields of keto-acids obtained by the degradation of citrate were observed to vary from bacterial strain to a strain.

5) The presence of a large amount of succinate brought about a striking inhibition on the action of isocitritase while no inhibiting effect was observed on the action of citritase.

6) The action of either isocitritase or citritase was not influenced by the presence of antibiotics (dihydrostreptomycin, chloramphenicol and tetracycline) and other inhibitors such as 2,4 dinitrophenol, NaN_3, NaF, *o*-phenanthroline and $\alpha\alpha'$-dipyridyl.

The authors wish to express their thanks to Dr. Y. Kuroiwa and Mr. Y. Horie of Kirin Research Institute for kindly supplying them with the chemicals used in this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 6, p. 489~492, 1959]

Microbiological Studies of *Coli-aerogenes* Bacteria
Part IX. On the Mechanism of Assimilation of Ammonia

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Received February 3, 1959

Aspartase activity, a combined reaction of aspartase and aspartic-glutamic transaminase and a coupling reaction between isocitric dehydrogenase and glutamic dehydrogenase were investigated in the bacteria of *coli-aerogenes* grown on the various media such as bouillon, citrate-bouillon and glucose-ammonium nitrogen. Aspartase activity was observed to be extremely high in all cases and furthermore it was ascertained that the combined reaction of aspartase and aspartic-glutamic transaminase occurred at an exceedingly high rate, while the coupling reaction between isocitric dehydrogenase and glutamic dehydrogenase occurred at a very much low rate when the fresh cell extracts were incubated without the addition of triphosphopyridine nucleotide to the reaction mixture. Isocitritase was operative even under such a condition where the fresh cell extracts were incubated in the presence of added ammonium ion. From this it was strongly suggested that aspartase or the combined system of aspartase and transaminase might play a principal role in the assimilation of ammonium nitrogen.

INTRODUCTION

In the investigations so far described in this series, the authors have demonstrated that the bacteria of *coli-aerogenes* possess an exceedingly high ability to produce α-ketoglutaric acid from a variety of carbonaceus substances including carbohydrate, pyruvic, lactic and C_4-dicarboxylic acids such as succinic, fumaric, malic and oxalacetic acids[1~5]. Moreover, it has been found that if C_6-tricarboxylic acids are present as a

TABLE I. FORMATION OF ASPARTIC ACID BY BACTERIA OF *Coli-aerogenes*

Bacterial strains	G–2 strain of *E. coli* grown anaerobically on bouillon	G–2 strain of *E. coli* grown aerobically on glucose ammonium N		B–2 strain of *A. aerogenes* grown aerobically on glucose-ammonium N
Bacterial cells used (mg)	Washed cells	Dried cells	Washed cells	Washed cells
	10	10	10	10
Na-fumarate added (μM)	450	450	400	400
NH_4Cl added (μM)	500	500	400	400
Total volume (ml)	5	5	5	5
Time of incubation (hours)	30	25	20	20
Aspartate produced (μM)	250	246	200	170

sole source of carbon, either little or no formation of α-ketoglutaric acid occurs by the bacteria of *coli-aerogenes*[5~7], but the addition of triphosphopyridine nucleotide to a mixture containing the fresh cell extract brings about an increase in the yield of α-ketoglutaric acid with citric acid[6,7], while the bacterial cells reveal a high potency of degradating citric acid into glyoxylic and succinic acids[5~7]. Thus, it has strongly been indicated that the bacteria of *coli-aerogenes* actually possess an enzyme isocitric dehydrogenase, but the physiological activity of the enzyme within the cells is extremely low owing to the dificiency of coenzyme triphosphopyridine nucleotide within the cells, and that another enzyme isocitritase which is independent upon the coenzyme may play a principal role in the oxidation of the tricarboxylic acids[5~7]. Such consideration has directed the authors' attention towards the problems concerning the assimilation of ammonia in the bacteria of *coli-aerogenes*. The methods of enzymic and fermentative preparations of glutamic acid have recently been reported by several workers[8~15], and consequently, it has frequently been supported that the reductive amination of α-ketoglutaric acid may be the main reaction of the synthesis of glutamic acid[8,11,15,16].

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EXPERIMENTAL AND RESULTS

Experimental procedures were performed by methods mentioned in the previous paper, except that amino-acids were determined by paper-partition chromatography[17] or with decarboxylase preparations.[18,19].

Occurrence of Aspartase. Quastel and Woolf[20] and Woolf[21] reported on the fact that in *E. coli* the synthesis of aspartic acid took place with ammonium nitrogen and fumaric acid. The authors have tested the activity of aspartase in the bacterial cells of *coli-aerogenes* grown under the following conditions of growth: (1) washed cells of G–2 strain of *E. coli* grown anaerobically for 4 days at 37°C on a bouillon containing 1% each peptone and chrysalis extract and 0.5% NaCl; (2) washed or dried cells of *E. coli* (G–2 strain) and *A. aerogenes* (B–2 strain) grown aerobically at 30°C for 2~4 days

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on a glucose-ammonium nitrogen medium which is used in the case of α-ketoglutaric acid-fermentation. The reaction mixtures (5 ml, pH 7.4) contained 50 μM phosphate, 400~450 μM Na-fumarate, 400~500 μM NH_4Cl and 10 mg the bacterial cells, and the incubations were carried out in the presence of toluol at 37°C under static conditions. From the results of experiments given in Table I, it has been observed that the formation of aspartase in *coli-aerogenes* takes place with either growth-media containing carbohydrate or bouillon-media not containing carbohydrate and that the bacteria also reveal an exceedingly high potency to produce a large amount of the enzyme within the cells.

Activities of Isocitric-glutamic Dehydrogenase System and Aspartic-glutamic Transaminase.

The authors have also investigated the metabolic relation between isocitric dehydrogenase and glutamic dehydrogenase and that between aspartase and aspartic-glutamic transaminase. Experiments carried out with both sonically treated cells and cell-free extracts of *coli-aerogenes* were as follows: a cell of G–2 strain of *E. coli* was grown aerobically on a glucose-ammonium nitrogen medium and that of B–2 strain of *A. aerogenes* grown aerobically on a citrate-bouillon medium in the same manner as already mentioned in the previous papers, and these washed cells were disintegrated by treatment with a 10 kc. Raytheon oscillator for 15~25 minutes. The experiments on the coupling reaction between isocitric dehydrogenase and glutamic dehydrogenase were carried out in the presence or absence of added triphosphopyridine nucleotide (TPN) with Na-citrate, ammonium chloride and sonically treated cells of *A. aerogenes* or fresh cell extracts of *E. coli*, while the combined reaction of aspartase and transaminase were carried out with Na-fumarate, ammonium chloride, Na-α-ketoglutarate and fresh cell-free extracts. The results of experiments with G–2 strain of *E. coli* grown on glucose are shown in Table II in which incubations were carried out anaerobically (in vacuo) at 37°C in a Thunberg tube with the media containing 200~600 μM phosphate buffer (pH 7.5), 10 μM $MgSO_4$ in case of exp. nos. I~IV, the indicated amounts of additions, 1 ml the cell-extracts prepared from 35 mg cells and water to make 3~9 ml. The results of experiments with B–2 strain of *A. aerogenes* are also shown in Table III, in which incubations were anaerobically carried out at 37°C in a Thunberg tube with the media containing 100 μM phosphate buffer (pH 7.5), 10 μM $MgSO_4$ in case of exp. nos. I and II, the indicated amounts of additions, 1 ml the disrupted cells or the cell extracts and water to make 3 ml. From the results shown in Tables II and III, it will be seen that both the activity of aspartase and that of aspartic-glutamic transaminase are exceedingly high in all of the cases tested, whereas, the treated cells or fresh cell extracts reveal an extremely low potency of synthesizing glutamate from ammonium ion and citrate. It should, however, be noted that the addition of coenzyme (TPN) to the reaction mixture brings about an increase in the yield of glutamate or α-ketoglutarate. Tables II and III also show that isocitritase present in the cell extracts is operative even under such conditions where ammonium ion is present, and there is not a glycine-glutamate transamination to be observed. The fact that the bacteria are easily obtained with the simple media and that the activity of aspartase is extremely high, will indicate the possibility to industrialize the preparation of aspartic acid with ammonium nitrogen and fumaric acid. Kitahara et al. have recently reported on the preparation of aspartic

Table II. Formation of Amino-acids or Keto-acids by Cell Extracts of *E. coli* (G–2 Strain) Grown Aerobically on Glucose-Ammonium Nitrogen

Exp. No.	I	II	III	IV	V	VI
Na-fumarate added (μM)	0	0	0	0	400.0	400.0
Na-citrate added (μM)	70.0	70.0	70.0	70.0	0	0
Na-α-ketoglutarate added (μM)	0	0	0	0	0	200.0
NH_4Cl added (μM)	0	0	170.0	170.0	500.0	500.0
Coenzyme II added (γ/ml)	0	35	0	35	0	0
Total volume (ml)	3	3	3	3	9	9
4 hours' incubation with 1 ml of cell extracts obtained from 35 mg bacterial cells						
Glyoxylate found (μM)	3.2	Trace	3.5	Trace	0	0
α-Ketoglutarate found (μM)	0.7	7.2	Trace	Trace		
Aspartate found (μM)	0	0	0	0	275.0	134.5
Glutamate found (μM)	0	0	7.2	40.5	0	71.5

TABLE III. FORMATION OF KETO- AND AMINO-ACIDS BY *A. aerogenes* (B-2 Strain) GROWN AEROBICALLY ON CITRATE-BOUILLON

Exp. No.	I	II	III	IV
Na-fumarate added (μM)	0	0	130.0	130.0
Na-α-ketoglutarate added (μM)	0	0	0	30.0
Na-citrate added (μM)	70.0	70.0	0	0
NH_4Cl added (μM)	130.0	130.0	130.0	130.0
Enzyme preparations	Sonically disrupted cells	Sonically disrupted cells	Cell extracts	Cell extracts
(mg cells used)	20	20	8	5
Coenzyme II added (γ/ml)	0	20	0	0
Total volume (ml)	3	3	3	3
4 hours' incubation				
Glyoxylate found (μM)	7.1	3.2	0	0
α-Ketoglutarate found (μM)	0.6	2.7	0	
Aspartate found (μM)	0	0	52.0	22.5
Glutamate found (μM)	Trace	6.1	0	12.2

acid by use of microorganisms[22].

In the previous papers, it has been demonstrated that the dried or washed (intact) cell which is an organized system, that is, in which a directive function of enzyme exists just as it is, excretes a large amount of glyoxylate and succinate in the external medium when incubation is carried out in the presence of citrate and a trapping agent such as semicarbazide. This may indicate that coenzyme triphosphopyridine nucleotide available for isocitric dehydrogenase within the cells exist only in a very much small amount, so that the coenzyme-dependent enzyme is not operative at such a high rate sufficient to effect complete removal of citrate, while another enzyme isocitritase which is independent upon the coenzyme (TPN) is operative at a high rate. This conception may also be supported from the results of experiments described in the present paper. In other words, it appears that a coupling reaction between isocitric dehydrogenase and glutamic dehydrogenase does not occur, in the bacteria of *coli-aerogenes*, at such a high rate sufficient to effect the complete transformation of citrate to glutamate.

Thus, the authors have strongly suggested that the primary reaction of assimilation of ammonium-nitrogen in *coli-aerogenes* proceeds by way of aspartase or the combined systems of aspartase and transaminase rather than by a coupling system of isocitric dehydrogenase and glutamic dehydrogenase.

SUMMARY

1) The activity of aspartase, a combined reaction of aspartase and aspartic-glutamic transaminase, and a coupling reaction between isocitric dehydrogenase and glutamic dehydrogenase were investigated with the bacterial cells or cell extracts of *coli-aerogenes* obtained under various conditions. The growth-media tested were such as bouillon-, citrat- bouillon-and glucose-ammonium nitrogen-media.

2) In all of the cases tested aspartase activity was observed to be extremely high.

3) It was ascertained that the combined reaction of aspartase and aspartic-glutamic transaminase by the cell extracts of the bacteria proceeded at an exceedingly high rate, whereas, a coupling reaction between isocitric dehydrogenase and glutamic dehydrogenase by the disrupted cells or the fresh cell extracts occurred at an exceedingly low rate.

4) It was found that isocitritase was operative even under such the conditions that the fresh cell extracts were incubated in the presence of addition of citrate and ammonium ion.

5) The addition of coenzyme triphosphopyridine nucleotide to a mixture containing the fresh cell extract brought about an increase in the yield of glutamate or α-ketoglutarate.

6) The mechanism of assimilation of ammonia in the bacteria was discussed.

This investigation has been supported in part by the Ajinomoto Co., Inc., to which the authors' thanks are due.

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[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 6, 493~498, 1959]

Biochemical Studies on "Bakanae" Fungus. Part 51.
Chemical Structure of Gibberellins. Part XVII.

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Received December 17, 1958

The outline of this report[1] has already been published in this Journal as a communication to the Editors. The relation among four isomers, gibberellin A_1, gibberellin C, pseudogibberellin A_1 and isogibberellin A_1 was clarified. The partial structure of gibberellin C is presented.

In the previous papers, three isomers of gibberellin A_1[2], i.e. gibberellin C[2,3], pseudogibberellin A_1[2], and isogibberellin A_1[4], have been reported. The present paper deals with the relation between these isomers.

When gibberellin A_1 was treated with mineral acid (20% H_2SO_4 or 10% HCl), gibberellin C was obtained in a 80% yield. By treating gibberellin C with sodium acetate and acetic anhydride, monoacetyl gibberellin C was obtained. Monoacetyl gibberellin C monomethyl ester which was obtained by treatment with diazomethane shows no OH streching vibration band in its infrared spectrum. Gibberellin C shows a maximum peak at 280 mμ (ε 30) in its ultraviolet spectrum which is characteristic for the carbonyl group and gives 2,4-dinitrophenylhydrazone. The existence of γ or δ-lactone and carboxyl group was confirmed by 1760 cm^{-1} and 1720 cm^{-1} absorption bands in its infrared spectrum. On the hydrogenation over Adams' catalyst, gibberellin C did not absorb hydrogen. From these results, it was concluded that gibberellin C has one hydroxyl, one ketonic carbonyl, one lactone and one carboxyl group, and that one OH (perhaps tertiary) and exocyclic methylene in gibberellin A_1 are converted to the one ketonic carbonyl group in gibberellin C on acid treatment of gibberellin A_1.

When gibberellin C was boiled with 0.1N NaOH for 30 min., and the reaction solution was backtitrated with 0.1N HCl, it was noticed that 1.8 moles of NaOH is consumed yielding isogibberellin A_1 from the acidified solution as a resulting product. Under the same condition, isogibberellin A_1 consumed 1.8 moles of alkali and from the acidified solution, original isogibberellin A_1 was recovered. This fact indicates the existence of one lactone and one carboxyl group in isogibberellin A_1. Its ultraviolet spectrum was quite similar to that of gibberellin C, showing the existence of a carbonyl group (285 mμ ε 30). Thus it was found that gibberellin C and isogibberellin A_1 have the same functional groups, and they were considered to be an epimer each other.

When gibberellin A_1 methyl ester was kept with 0.01N NaOH for 48 hours at 30°, gibberellin A_1 and pseudogibberellin A_1 were isolated by the chromatography of silicic acid. At that time, only one mole of alkali was consumed. Pseudogibberellin A_1 absorbed one mole of hydrogen on catalytic reduction, giving pseudodihydrogibberellin A_1. Its infrared spectrum shows the existence of the same with those of gibberellin A_1. Dihydrogibberellin A_1 gave

1) N. Takahashi, Y. Seta, H. Kitamura, A. Kawarada and Y. Sumiki, This Bulletin, **21**, 75 (1957).
2) N. Takahashi, A. Kawarada, H. Kitamura, Y. Seta, M. Takai, S. Tamura and Y. Sumiki, *ibid.*, **19**, 267 (1955).
3) A. Kawarada, H. Kitamura, Y. Seta, N. Takahashi, M. Takai, S. Tamura and Y. Sumiki, *ibid.*, **19**, 278 (1955).
4) T. Yabuta, Y. Sumiki, K. Aso, T. Tamura, H. Igarashi and K. Tamari, *J. Agr. Chem. Soc. Japan*, **17**, 894 (1940).

pseudodihydrogibberellin A_1 by the treatment of 0.01N NaOH, consuming only one mole of alkali and, this fact shows that the lactone ring in dihydrogibberellin A_1 is not opened in this condition. The changes caused by alkali, i.e., gibberellin A_1 to pseudogibberellin A_1, dihydrogibberellin A_1 to pseudodihydrogibberellin A_1, are considered to be the epimerization. When dihydrogibberellin A_1 was boiled with 0.5N NaOH for two hours, two moles of alkali were consumed, giving the pseudodihydrogibberellin A_1 mentioned above. This result indicates that the opening and closing of the lactone ring in pseudodihydrogibberellin A_1 are reversible.

When pseudogibberellin A_1 was treated with 30% H_2SO_4, isogibberellin A_1 was obtained in a 80% yield. Thus the relation among the four isomers was clarified and is summarized in Fig. 1.

gibberellin A_1	$\xrightarrow{OH^-}$	pseudogibberellin A_1
$C_{19}H_{24}O_6$ d.p. 232~5°		$C_{19}H_{24}O_6$ d.p. 225~7°
2 –OH		2 –OH
1 –COOH		1 –COOH
1 –COO–	H^+	1 –COO–
1 double bond		1 double bond
gibberellin C	$\xrightarrow{OH^-}$	isogibberellin A_1
$C_{19}H_{24}O_6 \cdot H_2O$ d.p. 252~4°		$C_{19}H_{24}O_6$ d.p. 260~2°
1 –OH		1 –OH
1 =CO		1 =CO
1 –COOH		1 –COOH
1 –COO–		1 –COO–
no double bond		no double bond

FIG. 1.

In the previous communication[1,2], we reported on the possibility of epimerization of α-hydrogen of carboxyl or the lactone group. But we have now reached a different conclusion that the secondary hydroxyl group participates in this epimerization, according to the fact that the oxidation product of pseudodihydrogibberellin A_1 was identical with that of dihydrogibberellin A_1[5,7]. Recently, Cross et al.[6] reported that the secondary hydroxyl group of gibberellic acid or gibberellin A_1 is easily epimerized.

We next examined the mechanism of rearrangement by acid, i.e. gibberellin A_1 to gibberellin C, pseudogibberellin A_1 to isogibberellin A_1. It was found that a change similar to the above-mentioned occurs in the bromination of gibberellin A_1. When gibberellin A_1 was brominated with dioxane-bromine complex at $-5°$, the monobromogibberellin A_1 was obtained, but dibromogibberellin A_1 could not be obtained. Monobromogibberellin A_1 shows a maximum peak at 285 mμ in its ultraviolet spectrum, the position of which is the same as that of gibberellin C and a new absoption band at 1740 cm^{-1} in infrared spectrum which was assigned to a five membered ring ketone. On the hydrogenation over Adams' catalyst in neutral medium, monobromogibberellin A_1 did not absorb hydrogen, but in the alkaline medium it was readily debrominated, giving the gibberellin C. On the other hand, monobromogibberellin A_1 was also converted to gibberellin C by boiling with zinc in acetic acid for 2 hours. The above results indicate that the double bond of gibberellin A_1 disappeared and a ketonic carbonyl is newly formed on the bromination of gibberellin A_1. Thus as the change of functional groups of gibberellin A_1 by the bromination is quite similar to that of the acid rearrangement of gibberellin A_1, we concluded that bromination and acid rearrangement occur in the same mechanism in which carbonium-cation-intermediate and its rearrangement are involved. If we accept (I) or (II) as the partial structure of gibberellin A_1 and the mechanism of rearrangement, four partial structures (IIIa), (IIIb), (IVa) and (IVb) are proposed to gibberellin C and four formulae (IIIa′), (IIIb′), (IVa′) and (IVb′) to monobromogibberellin A_1, as shown in Fig. 2.

As it has been described in our previous report[7,8] on hydrogenation by Se, gibberellin A_1

5) H. Kitamura, Y. Seta, N. Takahashi, A. Kawarada and Y. Sumiki, This Bulletin, **21**, 71 (1957).
6) B. E. Cross, J. F. Grove, J. MacMillan and T. C. Mulholland, Proceedings of the Chemical Society, August, 1958.
7) H. Kitamura, Y. Seta, N. Takahashi, A. Kawarada and Y. Sumiki, This Bulletin, **23**, 408 (1959).
8) Y. Seta, N. Takahashi, A. Kawarada, H. Kitamura and Y. Sumiki, This Bulletin, **23**, 412 (1959).

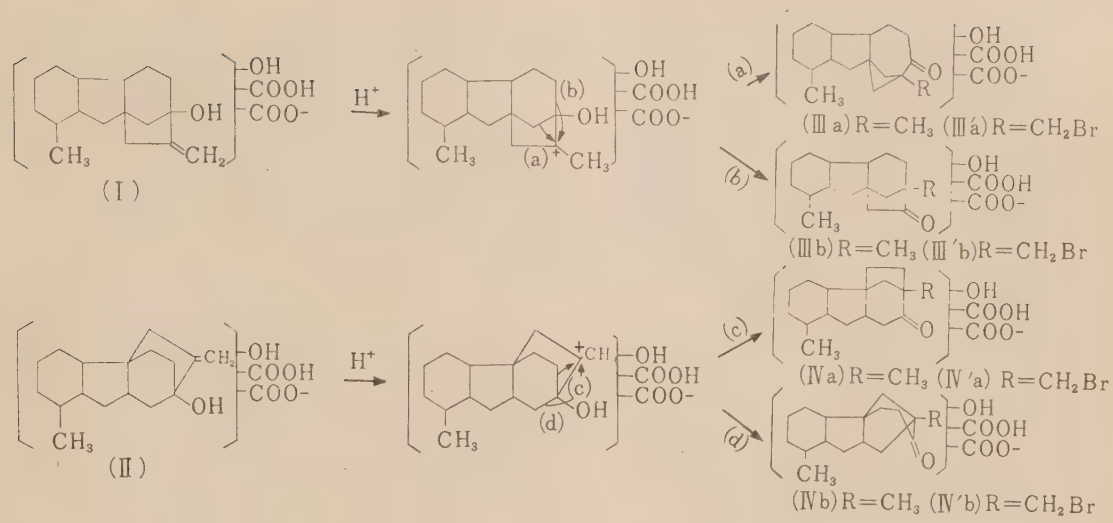

FIG. 2.

and C gave the same product, 1 : 7 dimethylfluorene, and no other hydrocarbon was isolated in the crystalline form. This result can be interpreted by the structure (I) as gibberellin A_1 and the structure (IIIb) as gibberellin C successfully, other formulae being excluded. Moreover, the fact that monobromogibberellin A_1 is not dehydrobrominated in boiling collidine can also be interpreted by the proposed formulae (IIIb') as monobromogibberellin A_1.

By SeO_2 oxidation of gibberellin C, a pale yellow α-diketone $C_{19}H_{22}O_7 \cdot H_2O$ was obtained which gave a positive ethylene diamine test. This fact shows the existence of active methylene group adjacent to the carbonyl group.

When gibberellin C was treated with an excess of bromine in acetic acid at 60°, only one mole of bromine was consumed no more bromine being absorbed. If gibberellin C has such a ring ketone system as shown in structure (IIIb), the substitution of a second bromine atom might be hindered sterically.

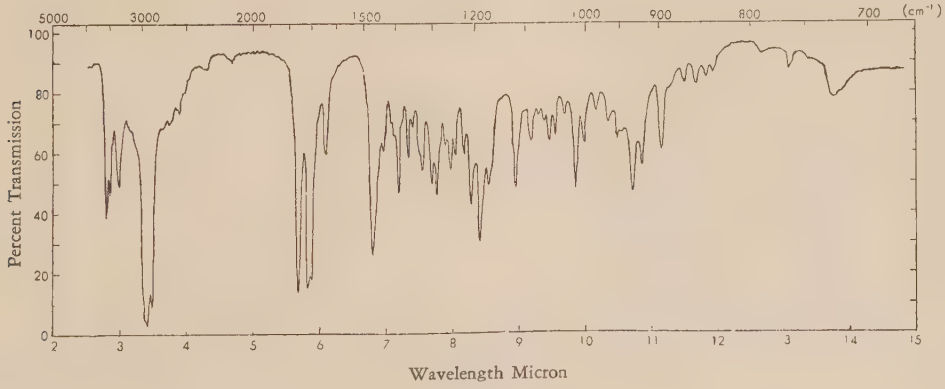

FIG. 3.—(1) Gibberellin C

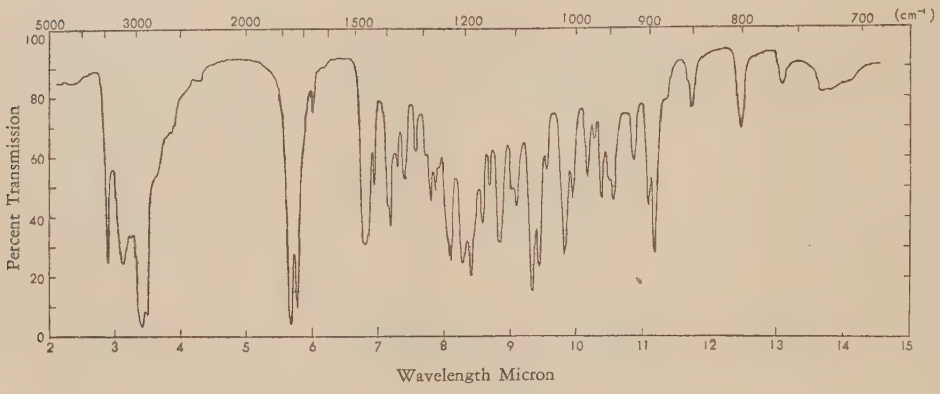

Fig. 3.—(2) Pseudo-gibberellin A_1

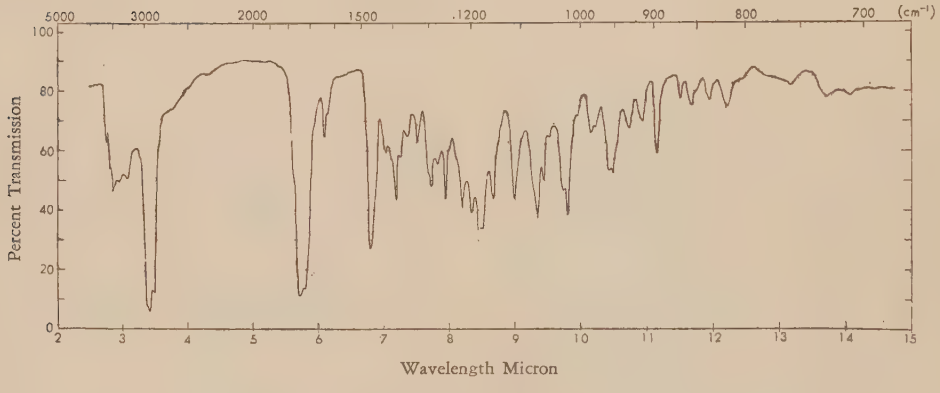

Fig. 3.—(3) Isogibberellin A_1 (hydrate)

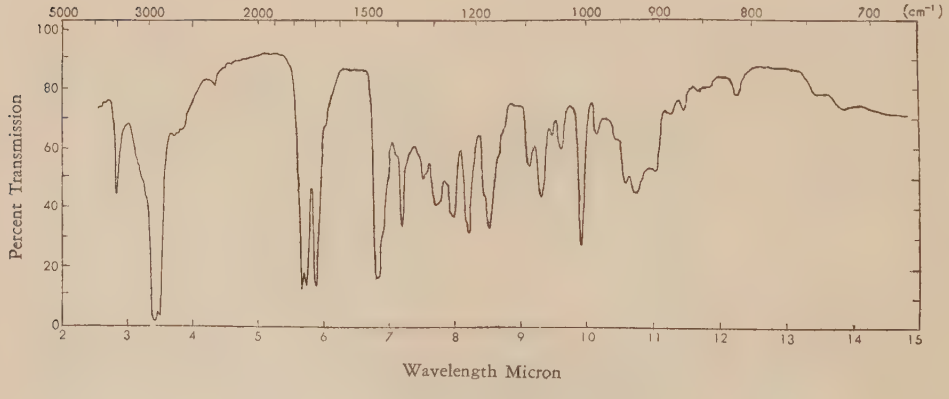

FIG. 3.—(4) Monobromogibberellin A_1

Cross et al. [9,10] proposed a similar partial structure of gibberic acid which is the acid degradative product of gibberellic acid (gibberellin A_3), from the view point of the same mechanism of acid rearrangement. Infrared spectra of gibberellin C, isogibberellin A_1, pseudogibberellin A_1 and monobromogibberellin A_1, are summarized in Fig. 3.

EXPERIMENTAL

1) Derivative of gibberellin C

a) Monoacetyl gibberellin C: A sample of 100 mg of gibberellin C was boiled for one hour with anhydrous sodium acetate and acetic anhydride. The mixture was evaporated in vacuo and treated with water. Needles crystallized out, yield, 80mg. It was recrystallized from ethyl acetate-ligroin, m.p., 265~7°. Found: C, 65.02; H, 6.73; Calcd. for $C_{21}H_{26}O_7$: C, 64.60; H, 6.71.

b) Monoacetyl gibberellin C monomethyl ester: Monoacetyl gibberellin C in methanol solution was treated with an excess of diazomethane. The crystal obtained on evaporation of the solvent was recrystallized from ethyl acetate-ligroin, m.p., 200°. Found: C, 65.80; H, 7.51; Calcd for $C_{22}H_{28}O_7$: C, 65.33; H, 6.98.

2) Isogibberellin A_1

a) An amount of 150 mg of gibberellin C was boiled with 150 ml of 0.1 N NaOH for 30 min.. From the acidified reaction mixture, isogibberellin A_1 was precipitated, yield, 120 mg. Recrystallization from ethanol-ligroin gave needles, d.p. 260~2°. It contained one mole of crystallization water which was removed in vacuo at 100° over P_2O_5. Found: C, 65.27; H, 6.87; Calcd. for $C_{19}H_{24}O_6$: C, 65.60; H, 6.94.

b) One hundred mg of pseudogibberellin A_1 was boiled with 30% H_2SO_4 for 30 min.. From the cooled solution, isogibberellin A_1 precipitated. This crystal was identical in all respects with isogibberellin A_1 obtained from alkali treatment of gibberellin C.

c) Isogibberellin A_1 methyl ester: When isogibberellin A_1 was treated with an excess of diazomethane, methyl ester was obtained, d.p., 270~2°. Found: C, 64.51; H, 7.03; Calcd. for $C_{20}H_{26}O_6$: C, 64.60; H, 6.71.

3) Pseudodihydrogibberellin A_1

a) Pseudogibberellin A_1 was hydrogenated over Adams' catalyst in methanol and one mole of hydrogen was absorbed. Upon evaporation of the solvent and recrystallization from ethanol-ligroin, pseudodihydrogibberellin A_1 was obtained, d.p. 290~5°.

b) One hundred and fifty mg of dihydrogibberellin A_1 was boiled with 50 ml of 0.1 N NaOH for 30 min.. The reaction solution was acidified and extracted with ether continuously. About 80 mg of crystal precipitated from ether, dp. 292~5°. Its infrared spectrum was identical with that of pseudodihydrogibberellin A_1 which was obtained from the hydrogenation of pseudogibberellin A_1. The same result was obtained by treatment of 0.5 N NaOH for 2 hours. At that time it was noticed that 1.8 moles of alkali are consumed by back titration.

c) One hundred and fifty mg of pseudodihydrogibberellin A_1 was dissolved in 10 ml of acetic acid. Then, 1.3 moles of CrO_3 was dissolved in 5 ml of acetic acid. This solution was added dropwise to the sample solution at 60~65° for 30 min., the solution colour changing to green. The solvent was evaporated in vacuo and the residual matter was treated with water and extracted with ether continuously. On evaporation of ether and recrystallization from ethyl acetate-ligroin, a crystal of d.p. 254° (60 mg) was obtained. Its infrared spectrum was identical with the oxidation product of dihydrogibberellin A_1.

4) Debromination of monobromogibberellin A_1

a) Two hundred mg of monobromogibberellin A_1 was dissolved in 150 ml of 0.1 N NaOH and hydrogenated over Pd-$CaCO_3$ catalyst. One mole of hydrogen was absorbed. After the catalyser was filtered off, the reaction mixture was acidified and extracted with ethyl acetate. The crystal obtained on evaporation of the solvent was recrystallized from ethyl acetate-ligroin, d.p., 254~6°, yield 110 mg. Infrared spectrum of this crystal was identical with that of gibberellin C and the mixed melting point with gibberellin C was not depressed.

b) Two hundred mg of monobromogibberellin A_1 in acetic acid was boiled with zinc for 2 hours. Zinc and zinc acetate were filtered off and the filtrate was evaporated. The residual solid was extracted

9) B. E. Cross, J. F. Grove, J. MacMillan and T. C. Mulholland, *Chem. and Ind.*, **36**, 954 (1956).

10) B. E. Cross, J. F. Grove, J. MacMillan and T. C. Mulholland, *J.C.S.*, **1958**, 2520.

with ethyl acetate and the solvent was removed in vacuo. A quantity of 80 mg of crystal which decomposed at 254~6° was obtained from recrystallization of ethyl acetate-ligroin. The identity of this crystal with gibberellin C was confirmed by comparison of their infrared spectra and by mixed melting point.

c) Monobromogibberellin A_1 was boiled in collidine for one hour. After collidine was evaporated, an unchanged original substance was recovered by recrystallization of residual syrup, no debromo-compound being obtained.

5) **Seleniumdioxide oxidation of gibberellin C**

An ethanol solution of 150 mg of gibberellin C in a sealed tube was heated with 200 mg of seleniumdioxide at 130~5° for three hours. About 140 mg of selenium precipitated. Neutral and acidic fractions were separated in the usual way. One hundred mg of the neutral fraction was subjected to alumina column chromatography. Elution of 5% ethyl acetate-benzene solution gave 50 mg of crystal, mp., 206~7°. It was concluded that this crystal was gibberellin C ethyl ester from its analytical datum and infrared spectrum. Found: C, 67.29; H, 7.35; Calcd. for $C_{21}H_{28}O_6$: C, 67.00; H, 7.50; 60 mg of acidic fraction gave a pale yellow crystal from ethanol-ethyl acetate-ligroin, dp. 240~8°. It gave a positive ethylene diamine test which was applicable for confirmation of α-diketone. Found: C, 60.65; H, 7.06; Calcd. for $C_{19}H_{22}O_7 \cdot H_2O$: C, 59.99; H, 6.36.

6) **Bromination of gibberellin C**

One hundred and fifty mg of gibberellin C in acetic acid was brominated with 1% bromine-acetic acid solution at 60°. One mole of bromine was relatively smoothly absorbed. Two moles of bromine were added and the solution was kept at 65° for two hours. Result of backtitration showed that only one mole of bromine is consumed. The reaction mixture was dried up and extracted with ethyl acetate. A portion of 120 mg of acidic fraction was subjected to silica gel chromatography (buffer pH 5.2 and silicic acid 15 g). Elution of 3% butanol-benzene solution gave two kinds of crystals. Fractions 2~5 (the volume of each fraction, 20 ml) gave monobromo-gibberellin C, (35 mg) d.p. 228~9°. Found: C, 53.43; H, 5.53; Br, 18.0; Calcd. for $C_{19}H_{23}O_6$ Br: C, 53.40; H, 5.39; Br, 18.74.

From fractions 7~10, another crystal, d.p. 202~5°, was isolated. The infrared spectrum of this crystal was different from that of monobromo-gibberellin C. Further characterization was not attempted on ground of negligible quantities.

Acknowledgement. The authors wish to express their thanks to the Central Institute of Japan Monopoly Corporation for performing the infrared spectra. This study was supported in part by the Grant in Aid for Agricultural Research defrayed by the Ministry of Education.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 6, p. 499~509, 1959]

Biochemical Studies on "Bakanae" Fungus. Part 52.
Chemical Structure of Gibberellins. Part XVIII.

By Yasuo Seta, Nobutaka Takahashi, Hiroshi Kitamura, Makoto Takai, Saburo Tamura and Yusuke Sumiki

Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo

Received December 17, 1958.

The outline of this report has already been published as a short communication[1,2] concerning the secondary hydroxyl group of gibberellin A_1 which was proved to be C–2 of its carbon skeleton. The relative position of the secondary hydroxyl group and lactone carbonyl are subsequently determined, and, finally, the attaching position of the lactone carbonyl was proved to be C–3 of its carbon skeleton.

As it had been described in our previous reports[3,4], the ozonolysis of gibberellin A_1 (G A_1) methyl ester (I) gave a monohydroxy-monocarbomethoxy-monolactonic-keto-carboxylic acid (III) $C_{19}H_{24}O_8$, via a ketol (II) $C_{19}H_{24}O_7$, which has a five-membered ring ketone, together with formaldehyde and formic acid. Thus, the presence of the $-C(OH)-C=CH_2$ group and a five-membered ring other than perhydrofluorene in G A_1 were indicated. The position of the ketogroup of (III), namely, the position of the tertiary hydroxyl group of G A_1, has been elucidated to be at C–7 of the carbon skeleton, by dehydrogenation with selenium, which afforded 1-methyl-7-hydroxyfluorene (VII). Further evidence for the relative position of the C–C double bond and tertiary hydroxyl group in G A_1, has been provided by degradation of G A_1 with mineral acid, in which it undergoes the Wagner-Meerwein rearrangement, affording gibberellin C (IV). Consequently, the partial structural formulae for these substances have been proposed to be in harmony with all the experimental results (Fig. 1).

1) Y. Seta, N. Takahashi, H. Kitamura, M. Takai, S. Tamura and Y. Sumiki, This Bulletin, **22**, 61 (1958).
2) Y. Seta, N. Takahashi, H. Kitamura and Y. Sumiki, *ibid.*, **22**, 429 (1958).
3) Y. Seta, H. Kitamura, N. Takahashi and Y. Sumiki, *ibid.*, **21**, 73 (1957).
4) N. Takahashi, Y. Seta, H. Kitamura, A. Kawarada and Y. Sumiki, *ibid.*, **21**, 75 (1957).

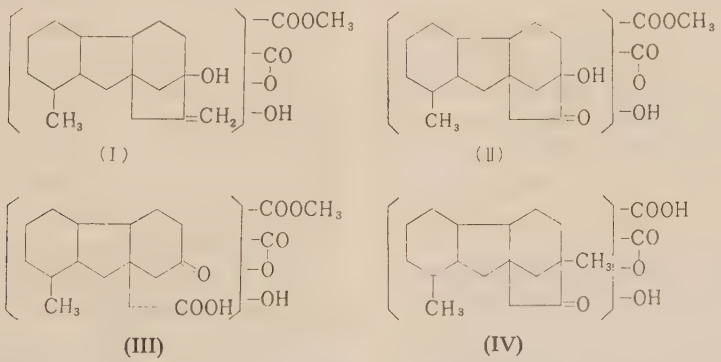

Fig. 1.

The structural relation between G A_1 and gibberellin A_3 was elucidated[5,6] as that gibberellin A_3 has the same configuration with G A_1, except that gibberellin A_3 has one more carbon-carbon double bond than G A_1. Therefore, the position of the carboxyl group of gibberellic acid (identical substance with our gibberellin A_3), unambiguously determined to be at C–9 by Cross et al.[7], who obtained (±) methyl-1,7-dimethyl-fluorene-9-carboxylate from gibberdionic acid mono-methylester by dehydrogenation, might be accepted for G A_1. Accordingly, the remaining problems to be clarified, are the determination of the position of secondary hydroxyl group and lactone.

In this paper, the authors wish to report on our experimental results for the determination of the secondary hydroxyl group and lactone carbonyl in G A_1. First, the position of the secondary hydroxyl group was proved to be C–2 of its carbon skeleton in the following way.

As it had been already pointed by several authors[8,9,10], cyclic ketones are readily converted to phenols by dehydrogenation. Therefore, dehydrogenation of the keto-derivatives, which were obtained from dihydro-G A_1 and gibberellin C by oxidation with chromic acid, was attempted to obtain corresponding fluorenols, but the results were less fruitful results. Dehydrogenation of the keto-derivative of dihydro-G A_1 at 280~300° gave a considerable amount of phenolic substance but could not be obtained in the crystalline form.

In the case of keto-derivative from gibberellin C, a crystalline phenolic substance $C_{17}H_{20}O_2$, was obtained. It gives a red coloration with ferric chloride and is almost insoluble on sodium bicarbonate solution, but is readily soluble in sodium hydroxide solution, showing strong blue fluorescence. In the infrared region, the product shows, among other absorption bands due to the phenolic group, an absorption band at 826 cm^{-1}, suggesting the presence of 1, 2, 3, 4, tetrasubstituted benzene ring. However, from the molecular formula of this substance it is clearly indicated that the elimination of ring D does not occur under such mild reaction conditions that a sufficient yield of phenolic substances are expected, and thus, the required fluorenol derivative should not be formed. Therefore, the ozonolysis product of G A_1 methyl ester, in which the ring D was already cleaved, was adopted subsequently as the starting material for dehydrogenation. Reduction of the ozonolysis product (III) with amalgamated zinc and hydrochloric acid afforded a dexo-product (V), $C_{19}H_{26}O_7$. It showed infrared absorption at 1750 cm^{-1} (shoulder) (lactone), 1718 cm^{-1} (ester carbonyl) and 1695 cm^{-1} (shoulder) (carboxyl) and no characteristic absorption in ultraviolet region due to the keto-group. Oxidation of a secondary hydroxyl group of this substance (V) with chromic acid was, unexpectedly, accompanied with stimultaneous decarboxylation, giving a neutral keto-derivative (VI), $C_{18}H_{24}O_5$. It seems that the carboxyl group from lactone was eliminated during oxidation, since the product (VI) showed infrared absorption at 1766 cm^{-1} (lactone), 1718 cm^{-1} (ester carbonyl) and 1706 cm^{-1} (6-membered ring ketone) and the presence of one carbomethoxyl group was confirmed by micro-analysis. But, from the relative position of lactone carbonyl and secondary hydroxyl groups, which should be dealt with later, it might be more reasonable to consider that during the process of the Clemmensen reduction, the original lactone ring was cleaved at first and the hydroxyl group thus formed, re-lactonized with the carboxyl at C–14 and then, the carboxyl from lactone was eliminated by the influence of ketone formed by oxidation of a secondary hydroxyl group.

This phenomenon gives suggestion concerning the position of the lactonic oxygen, that it may be at C–10 or C–11 of the carbon skeleton.

5) N. Takahashi, Y. Seta, H. Kitamura, and Y. Sumiki, This Bulletin, **21**, 327 (1957).
6) J. F. Grove, P. W. Jeffs and T.P.C. Mulholland, *J. Chem. Soc.*, **1958**, 1236.
7) B. E. Cross, J. F. Grove, J. MacMillan and T.P.C. Mulholland, *J. Chem. Soc.*, **1958**, 2520.
8) L. Ruzicka, *Helv. Chem. Acta*. **19**, 419 (1936).
9) D. A. Peak and R. Robinson, *J. Chem. Soc.*, **1936**, 759.
10) A. Harris, A. Robertson, and B. Whalley, *J. Chem. Soc.*, **1958**, 1799.

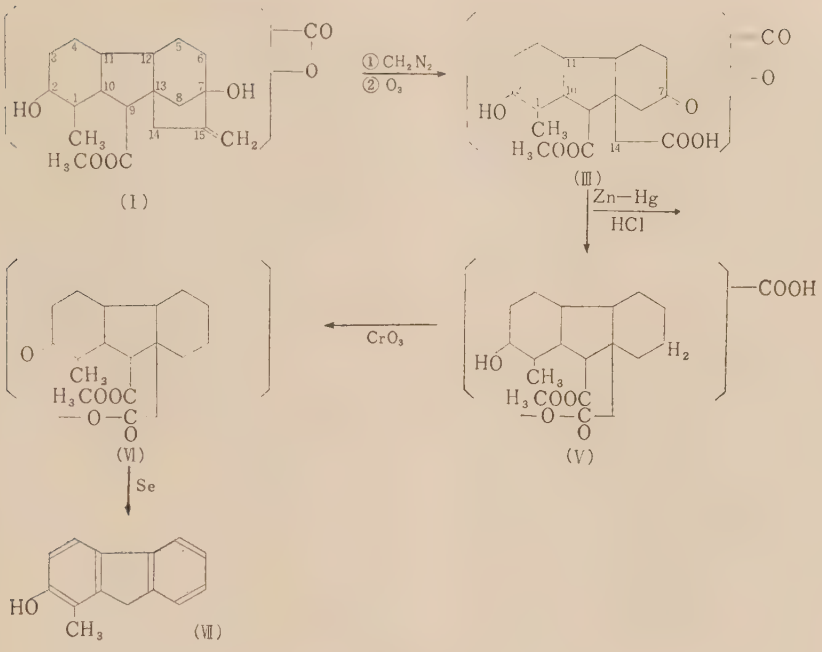

FIG. 2.

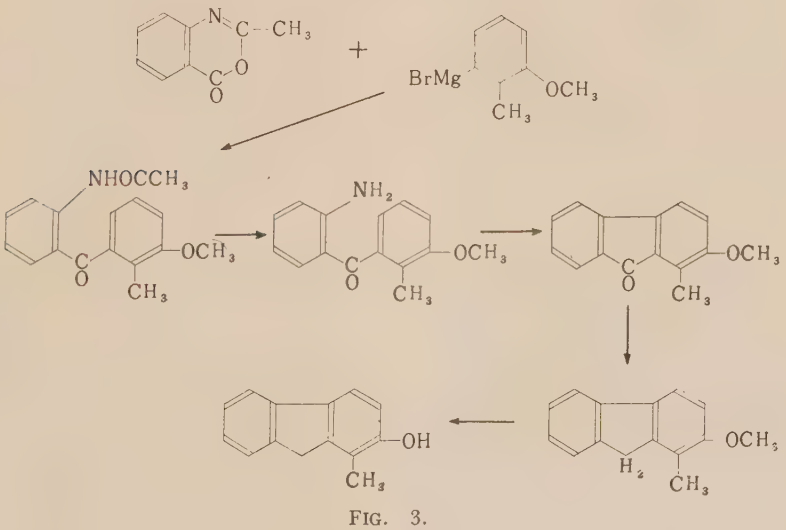

FIG. 3.

When this product (VI) is dehydrogenated with selenium at 320° a phenolic substance, $C_{14}H_{12}O$, was obtained. This had been proved to be identical with 1-methyl-2-hydroxy fluorene (VII) which was synthesized through the process shown in Fig. 3 in mixed m. p., ultaviolet and infrared spectra. The details of the synthesis of 1-methyl-2-hydroxy fluorene will be dealt with in our following paper.

The formation of the fluorenol clearly indi-

cates that the position of the keto-group of (VI), namely, the position of the secondary hydroxyl group of original G A_1, is C–2 of its carbon skeleton. Thus, the partial structure of G A_1 methyl ester can be shown as (I) in Fig. 2.

Subsequently, the dehydration of the secondry hydroxyl group of the ozonolysis product was attempted to obtain some structural information of ring A, especially in the neighborhood of C–2.

The methyl ester of the ozonolysis product of G A_1 gave, when dehydrated with phosphorus oxychloride and pyridine, a monoanhydro-product, $C_{20}H_{24}O_7$. Since the product shows no characteristic high-intensity absorption band at the ultraviolet region below 210 mμ, the existence of conjugated chromophoric system was excluded. In the infrared region, it showed absorption bands at 1758 cm^{-1} (lactone), 1730 cm^{-1} (ester carbonyl), 1704 cm^{-1} (6-membered ring ketone). Catalytic hydrogenation of this substance over platinum oxide gave a dihydro-compound, uptaking 1 mole of hydrogen. The presence of enol-lactone group might be excluded, since the product showed no infrared absorption band in the vicinity of 1800 cm^{-1} and was not altered by treatment with acid. Rearrangement of the ring system during dehydration reaction should not be considered, since dehydrogenation of the product and its dihydro-derivative with selenium gave the same 1-methyl-7-hydroxy fluorene. In our previous communication[1], though the formation of formaldehyde from this dehydration product by ozonolysis has been reported, it was confirmed from further investigations undertaken successively that the production of formaldehyde could not be detected. From the neutral reaction product, which was obtained by ozonolysis in about a 60% yield, a small amount of a crystalline substance was separated. Molecular formula for this product, $C_{19}H_{22}O_8 \cdot H_2O$ was given from analytical data. But whether this product is really the expected ozonolysis product or not, is open to queation, since the dehydrogenation of this substance gave 1-methyl-7-hydroxy fluorene.

In the next place, the relative position of lactone carbonyl with the secondary hydroxyl group was determined in the following manner. Previous investigations[5] described in this series have shown that, reduction of gibberellin A_1 methyl ester with lithium aluminum hydride afforded a pentaol* $C_{19}H_{32}O_5$, and this product resisted to periodic acid oxidation. However, this gives monoacetonyl monoanhydro compound (VIII), $C_{22}H_{34}O_4$, when its acetone solution is treated with sulfuric acid, indicating the presence of one 1,3-glycol linkage in its molecule.

Oxidation of dihydro-G A_1 with chromic acid in pyridine, gave C–2 keto-compound (IX), $C_{19}H_{24}O_6$. It shows infrared absorption at 1760 cm^{-1}, 1709 cm^{-1} and 1695 cm^{-1} due to lactone, 6-membered ring ketone and carboxyl, respectively. This keto-compound (IX) gave, when boiled with 25% sulfuric acid, a keto-monocarboxylic acid (X), $C_{18}H_{24}O_4$, as the main product, losing one mole of carbon dioxide, and an α,β-unsaturated ketone (XI), $C_{17}H_{24}O_2$, as the minor product, losing two moles of carbon dioxide. The product (X) showed infrared absorption at 1724 cm^{-1} and 1709 cm^{-1}, indicating the presence of a 6-membered ring ketone and carboxyl respectively, and the product (XI) showed infrared absorption at 1642 cm^{-1} and ultraviolet absorption at 247 mμ (ε, 9500) and 310 mμ (ε, 137) indicating the presence of the α,β-unsaturated ketone. But the absorption of lactone at 1760 cm^{-1} that existed in (IX), was lacking in both products (X) and (XI). Since the elimination of lactone could not be observed by the similar treatment of dihydro-G A_1 itself, this fact shows that the ketone at C–2 locates at the β-position towards the lactone carbonyl and facilitates delactonization. Furthermore, the double bond of the product (XI) is to be located at C–10$\sim$C–1, because the easiness of decarboxylation of the original carboxyl is attributable to the presence of such a conjugated

* The detail of this pentaol will be presented in our following paper, part 53 of this series.

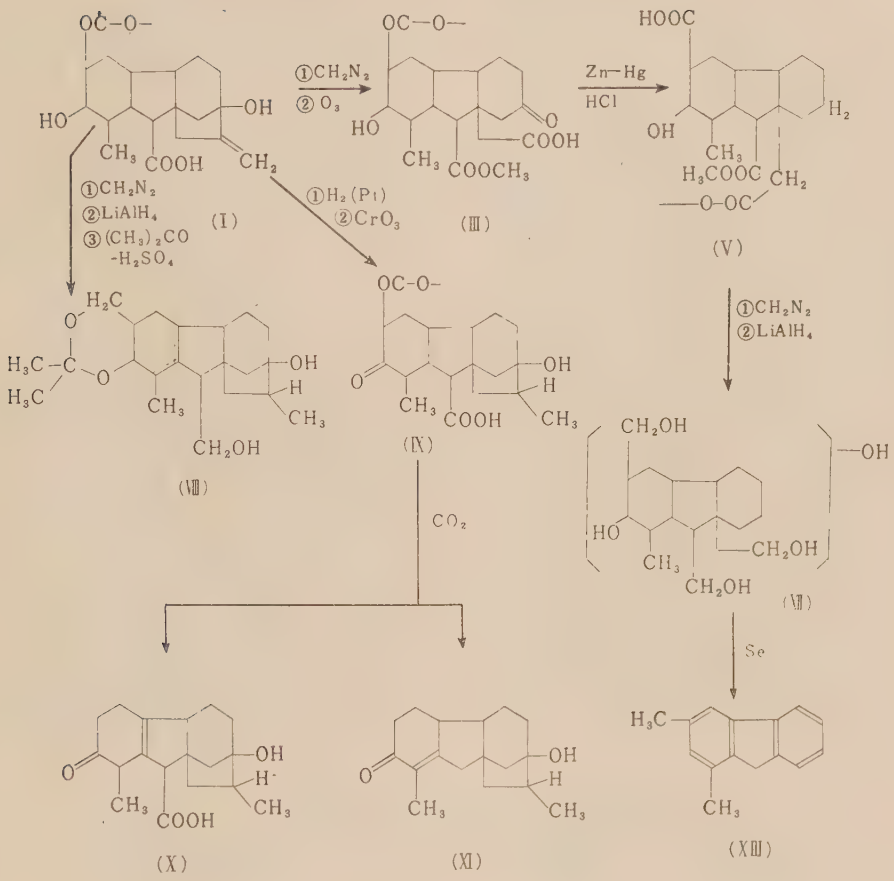

FIG. 4.

system as $-\overset{\overset{O}{\|}}{C}-C=C-$ at the β-position to this carboxyl (Fig. 4).

From these facts, it is ascertained that the lactone carbonyl should be attached to C–3 or C–1 of ring A.

Further, the position of the lactone carbonyl was determined from the following experimental results.

As already described, reduction of the ozonolysis product, $C_{19}H_{24}O_8$, of G A_1 metyl ester with amalgamated zinc and hydrochloric acid, afforded a deoxo-product (V), $C_{19}H_{26}O_7$. The methylester, $C_{20}H_{28}O_7$, of this compound was boiled with lithium aluminum hydride, carbomethoxyl and lactone carbonyl groups were reduced to hydroxymethyls, giving a pentaol (XII), $C_{18}H_{32}O_5$. Dehydrogenation of this product with selenium at 360~400° afforded a mixture of hydrocarbon m.p. 54~7°, which was considered to be a fluorene derivative from ultraviolet absorption spectrum. In the infrared region, it showed strong characteristic absorption bands at 855 cm^{-1}, 766 cm^{-1}, 755 cm^{-1} and 735 cm^{-1} and weak absorption bands at 790 cm^{-1}. After recrystallization five times from dilute ethanol, the melting point was raised to 94~7° and the absorption band at 790 cm^{-1} and 755 cm^{-1} completely disappeared. It showed m. p. 97~100° on admixture with the synthetic 1,3-

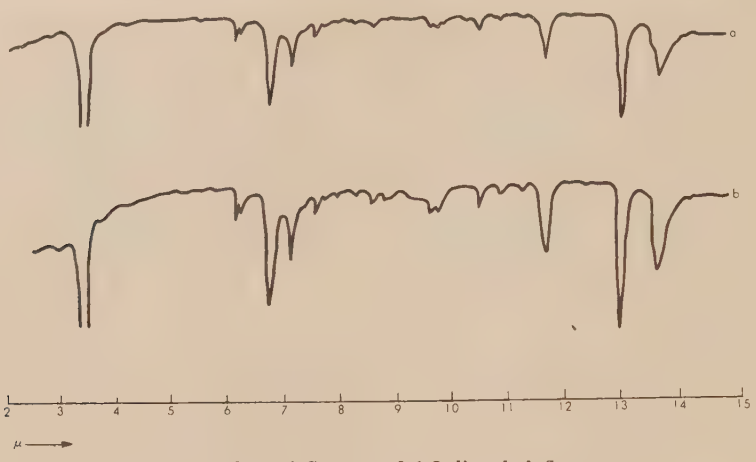

Fig. 5. Infra-red Spectra of 1,3-dimethyl fluorene

a. dehydrogenation product (recryst. 4 times)
b. synthetic specimen (Nujol mull)

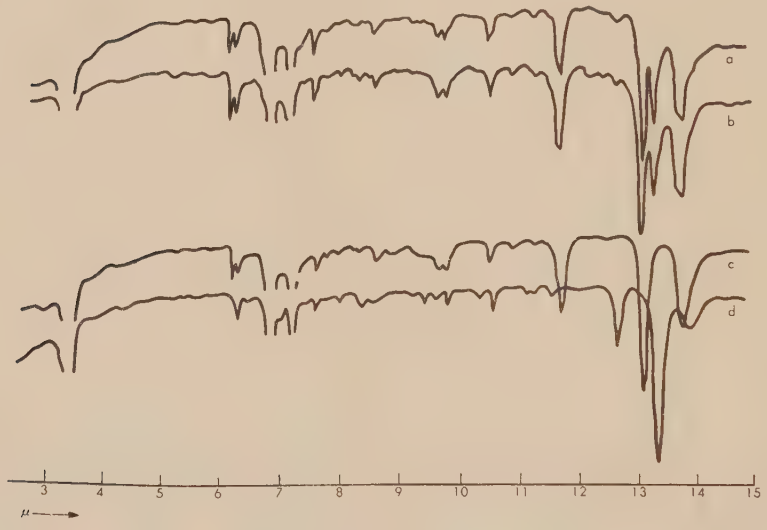

Fig. 6. Infra-red Spectra of

a. Synthetic mixture (1,3-dimethyl fluorene 80%, 1-methyl fluorene 20%)
b. Dehydrogenation product
c. 1,3-dimethyl fluorene
d. 1-methyl fluorene (Nujol mull)

dimethyl fluorene (XIII) (m.p. 99~100°) and was also shown to be identical with it by comparison of infrared and ultraviolet absorption spectra* (Fig. 5).

Absorption bands of the original mixture at 855 cm^{-1}, 766 cm^{-1} and 735 cm^{-1} were indicated to be originated from that of 1,3-dimethyl fluorene. On the other hand, the absorption

* The details of the synthesis of 1,3-dimethyl fluorene and 1,3,9-trimethyl fluorene shall be dealt with in our following report.

bands at 755 cm^{-1} and 790 cm^{-1}, may be due to that of 1-methyl fluorene, since 1-methyl fluorene has a characteristic strong absorption band at 755 cm^{-1} and a rather weak one at 790 cm^{-1} and 730 cm^{-1}. The amount ratio of 1,3-dimethyl fluorene to 1-methyl fluorene in the original mixture was shown to be about 80 to 20, by preparing the synthetic mixture of 1,3-dimethyl fluorene and 1-methyl fluorene in vaious composition and comparison of their infrared spectrum with that of the original mixture (Fig. 6).

If the hydroxymethyl group, which was derived from lactone carbonyl, is attached to C–1 together with original methyl group, it will split off during dehydrogenation to give 1-methyl fluorene only, and 1,3-dimethyl fluorene should not be formed. Therefore, the 3-methyl substituent must originate from the hydroxymethyl at C–3 of pentaol (XII) which was formed from lactone carbonyl by lithium aluminum hydride reduction.

From these results described above, it is concluded that the position of the lactone carbonyl of A_1 must be at C–3 of ring A, and the partial structural formula for G A_1 can be presented as the formula (I) in Fig. 4 and the reaction procedures discussed above are to be illustrated as in Fig. 4.

Dehydrogenation of the product (XII) at relatively low temperature to obtain 9-substituted fluorene derivatives, by which further evidence for the position of the carboxyl group could be expected, were attempted at the same time.

When the reaction was carried out at 340°～360°, a considerable amount of a phenolic substance were obtained. It was also produced in the case of high temperature dehydrogenation but resulted in a poor yield. Molecular formula, $C_{14\sim15}H_{12\sim14}O$ was given for this product from analytical data, but this differed from 1-methyl-2-hydroxy fluorene. A mixture of fluorene hydrocarbon were also obtained, which was oily at room temperature but crystallized at 0°～10°. It was suggested that the infrared absorption peaks of this substance at 855 cm^{-1}, 778 cm^{-1}, 766 cm^{-1} and 732 cm^{-1} might be due to the presence of 1,3,9-trimethyl fluorene and the peaks at 793 cm^{-1}, 754 cm^{-1} and 742^{-1} cm might be due to 1,9-dimethyl fluorene, by comparison with the spectrum of a synthetic specimen (Fig. 7).

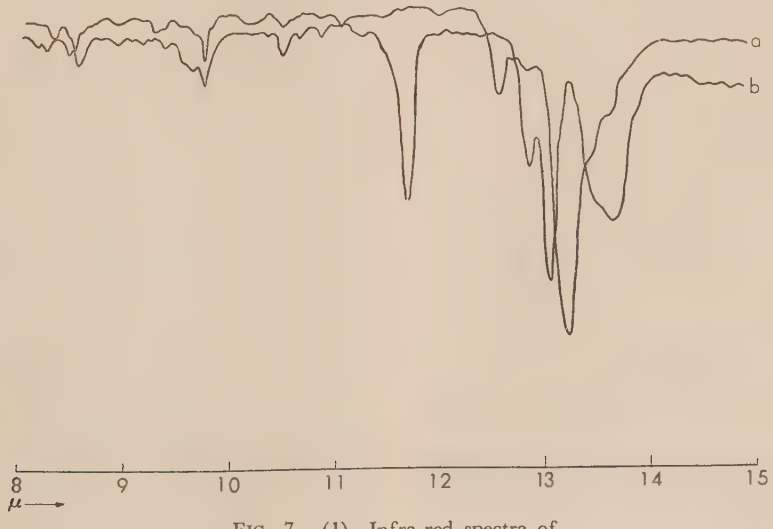

FIG. 7.—(1) Infra-red spectra of

a. 1,9-dimethyl fluorene
b. 1,3,9-trimethyl fluorene (Nujol mull)

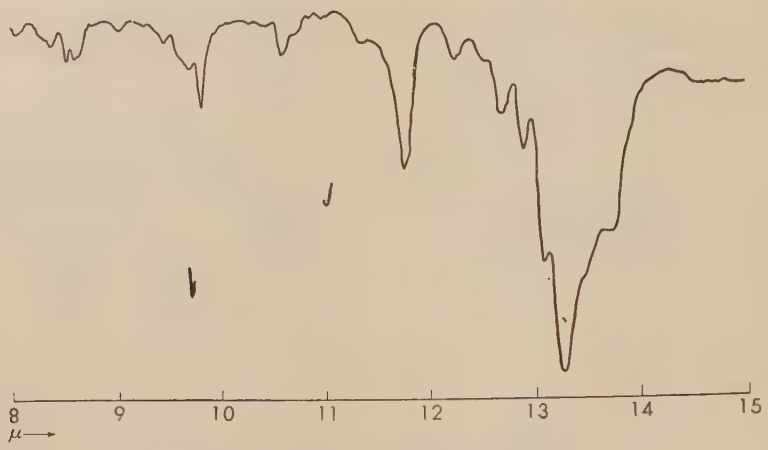

FIG. 7.—(2) Infra-red specrum of dehydrogenation Product (at 340~360°) (Nujol mull)

In spite of every attempt to separate the original mixture into each component were unsuccessful.

Although we could not obtain sufficient data to determine the position of lactonic oxygen from G A_1, it was determined to be located at C-10 of its carbon skeleton from the structure of gibberellin A_3 in connection with the position of its ethylenic linkage in ring A. Therefore, the structure of G A_1 must be formulated as shown in Fig. 8.

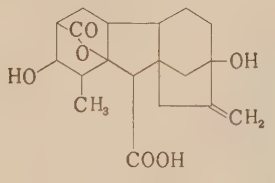

FIG. 8.

EXPERIMENTAL

Dehydrogenation of the keto-derivative of Gibberellin C.

Keto- gibberellin C (0.28 g) and selenium powder (0.5 g) were mixed and heated at 300~320° for 3.5 hrs.. The reaction mixture was extracted with ether and the ether layer was washed with 2% sodium bicarbonate solution and subsequently with 2% sodium hydroxide solution. The phenolic substance was recovered from the latter by acidification and extraction with ether. The etherial solution was evaporated and the product was distilled at 120~150° under 1 mm/Hg. The solidified distillate (54 mg) was dissolved in benzene and chromatographed over alumina (acid-washed Brockmann, 0.5 × 10 cm) and eluted with benzene, subsequently with benzene-ether (8 : 2 v/v), yielding long prisms, which sublimed at 190~220° and melted at 240~243° after recrystallization from benzene-ethanol-ligroin (Found C, 80.05; H, 7.45 Calcd. for $C_{17}H_{20}O_2$, C, 79.7; H, 7.8).

Reduction of the ozonolysis product (III) of G A_1 methyl ester with amalgamated zinc and hydrochloric acid.

Ozonolysis product (III) (1.1 g), amalgamated zinc (10 g flower zinc, 1 g $HgCl_2$, 0.5 ml. conc. hydrochloric acid and 10 ml water were shaken for 10 min. and washed by decantation) and 22% hydrochloric acid (50 ml) were boiled vigorously for 2.5 hrs. the aqueous layer was decanted from zinc, extracted continuously with ether for 72 hrs., and the extract was purified by partition chromatography of silicic acid, using 1 M phosphate buffer solution of pH 5.2 as the stationary phase and benzene-*n*-butanol (97 : 3 v/v) as the mobile phase. The product (V) (0.44 g) was obtained as colorless prisms, d.p. 252~254° after recrystallization from ethyl acetate-ligroin, $[\alpha]_D^{25}+46.2$ (c, 1.560 in MeOH) (Found C, 62.30; H 7.23; $-OCH_3$, 7.97. Calcd. for $C_{19}H_{26}O_7$, C, 62.3; H, 7.1; $-OCH_3$, 8.6).

The methyl ester, prepared with diazomethane,

crystallized from dil. methanol, prism, m.p. 154~156° $[\alpha]_D^{25}$+40.9 (c, 1.440 in MeOH) (Found. C, 63.27; H, 7.75; -OCH_3, 15.63. Calcd. for $C_{20}H_{28}O_7$, C, 63.2. H, 7.3; 2-OCH_3, 16.3).

Oxidation of the deoxo-product (V) with chromic acid.

Deoxo-product (V) (0.21 g) was dissolved in glacial acetic acid (15 ml), kept at 60~65° on the water bath and a solution of chromic acid (60 mg) in 90% acetic acid (6 ml) was gradually added for a period of 1 hr. Warming was continued for 1.5 hr. after completion of addition of the reagent, and methanol was then added to destroy excess chromic acid, acetic acid were evaporated in vacuo, water was added and it was extracted with ethyl acetate. After evaporation of solvent, the product (VI) was crystallized from dil. methanol as a prism, m.p. 173~174.5° $[\alpha]_D^{25}$+46.8° (c, 0.833 in MeOH) (Found. C, 66.93; H, 8.21; -OCH_3, 8.95 Calcd. for $C_{18}H_{24}O_5$, C, 67.5; H, 7.5 -OCH_3, 9.7) λ_{max} 282 mμ (ε, 30.1 in MeOH) 2,4-dinitro phenylhydrazone, yellow powder, d.p. 195°.

Dehydrogenation of the keto-ester (VI) with selenium.

The keto-ester (VI) (200 mg) was mixed with selenium powder (700 mg) and heated at 260° for 30 min., and the reaction temperature was raised to 305~320° and it was kept at that temperature for 2.5 hrs.. The mixture was extracted with ether, the extract washed with sodium bicarbonate solution, dried and evaporated. The residue thus obtained was distilled under 1 mm/Hg at 130~150° (bath temperature), the distillate (54 mg) was dissolved in benzene, chromatographed through a column of alumina (Brockmann, 0.5×10 cm) eluted with benzene and subsequently with benzene-ether (8:2 v/v), obtaining a crystal of m.p. 185~187°, after recrystallization from benzene-ligroin. It showed no depression on admixture with a synthetic 1-methyl-2-hydroxy fluorene, m.p. 185~187° (Found: C, 85.25; H, 5.99 Calcd. for $C_{14}H_{12}O$, C, 85.7; H 6.1).

Dehydration of the methyl ester of the ozonolysis product (III).

The methyl ester (110 mg) was dissolved in anhydrous pyridine (3 ml), phosphorus oxychloride (0.3 ml) was added and the mixture was heated at 100° for 30 min.. The reagents were then distilled off in vacuo, and the residue was diluted with water, acidified and extracted with ether. The extract (100 mg) was re-methylated with diazomethane, chromatographed through the column of alumina (Brockmann, 1×10 cm), and eluted with benzene-ethyl acetate (10:1 v/v). The product (45 mg) was obtained as needles and showed m.p. 135~136° after recrystallization from benzene-ligroin. $[\alpha]_D^{25}$−42.9 (c. 0.770 in MeOH) (Found: C, 64.06; H, 6.31; -OCH_3, 14.45 Calcd. for $C_{20}H_{24}O_7$, C, 63.8; H, 6.4; 2-OCH_3, 16.5) λ_{max} 290 mμ (ε, 37.7).

Catalytic hydrogenation of the dehydration product.

The product (60 mg) was dissolved in methanol and shaken with Adams's platinum (45 mg) in atmosphere of hydrogen. A quantity of 4.2 ml of hydrogen was absorbed in 1~2 min. (calcd. amount 3.6 ml). The reaction mixture was filtered to separate from the catalyst. The solvent was removed and the product was crystllized from ethyl acetate-ligroin as a needle, m.p.; 114~116° $[\alpha]_D^{25}$+27.2 (Found: C, 62.66; H, 6.98. Calcd. for $C_{20}H_{26}O_7$, C, 63.5; H, 6.8).

Treatment of the dehydration product with acid.

The dehydration product (64 mg) was dissolved in methanol (1 ml), 5% hydrochloric acid (8 ml) was added to it and it was refluxed for 1 hr.. The mixture was extracted with ethyl acetate and the extract was methylated with diazomethane, and then chromatographed through alumina (Brockmann) (1×7 cm). The starting material was recovered, showing m.p. and mixed m.p. 136° and was also shown to be identical by comparison of infrared spectra.

Dehydrogenation with selenium.

a) The dehydration product (0.4 g) and powdered selenium (1.2 g) were mixed and heated at 300~320° for 2.5 hrs.. The whole mixture was extracted with ether, and the extract was distilled by 3 mm/Hg at 180~220° (bath temperature). The distillate was chromatographed over alumina (0.5×10 cm) and eluted with benzene and subsequently with benzene-ether (9:1), yielding fine needles (22 mg) after recrystallization from ligroin, m.p. 162~163°. Mixed m.p. with 1-methyl-7-hydroxy fluorene (m.p. 166~167°), 164~167°. This identity was also shown by comparison of infrared spectrum.

b) An approximately similar procedure was applied for its hydrogenation product (0.20 g). The reaction product (12 mg) was shown to be 1-methyl-7-hydroxy fluorene by mixed m.p. and infrared spectrum.

Ozonolysis of the dehydration product.

The dehydration product (200 mg) was dissolved in

acetic acid (10 ml) and ozonized at 15~20° with an oxygen stream containing ca. 3% ozone for 1 hr., and steam-distilled immediately.

Formdimedone was not obtained from the neutralized distillate by the addition of dimedone solution. Non-volatile fractions were separated into neutral and acidic components by extraction with ethyl acetate. The neutral product (120 mg) was chromatographed over alumina (Brockmann, 1 × 15 cm) and eluted with benzene-ethyl acetate (10 : 1). The crystalline substance (40 mg) was eluted, showing m.p. 74~77° after recrystallization from dilute ethanol. (Found : C, 57.58 ; H, 5.92. Calcd. for $C_{19}H_{22}O_8 \cdot H_2O$ C, 57.6 ; H, 6.06). Viscous oil (20 mg) was subsequently eluted, and this was not obtained on crystalline form. But it reduced ammonium silver nitrate solution markedly, showing the presence of the aldehyde group.

Acetonyl compound of Lithium aluminum hydride reduction product of G A_1 methyl ester.

The reduction product (400 mg) was dissolved in acetone (50 ml), conc. sulfuric acid (2 ml) was added to this solution and the mixture was allowed to stand at 25° for 1.5 hrs.. Then, the reaction mixture was poured into 3% sodium bicarbonate solution (200 ml), extracted with ethyl acetate, and the extract (280 mg) was chromatographed over alumina (Brockmann, 1 × 10 cm) and eluted with benzene-ethyl acetate (5 : 1 v/v). A monoacetonyl-monoanhydro-compound (VIII) was obtained as needles, m.p. 137~139°, after recrystallization from ethyl acetate-ligroin. (Found : C, 72.81 ; H, 9.21. Calcd. for $C_{22}H_{34}O_4$, C, 72.89 ; H, 9.45).

Oxidation of dihydro-G A_1 with chromic acid-pyridine complex.

Dihydro-G A_1 (500 mg) was dissolved in pyridine (5 ml) and the solution was added to the mixture of chromic acid (500 mg) and pyridine (10 ml) and allowed to stand for 3~4 days at room temperature. Finally, methanol was added to destroy the excess of chromic acid, diluted with water, acidified and extracted continuously with ether. The extract (480 mg) was purified by partition chromatography of silicic acid, by use of a 1 M phosphate buffer solution of pH 5.4 as the stationary phase and benzene-*n*-butanol (97 : 3 v/v) as the mobile phase. The product (IX) was obtained as prisms, d.p. 256~258° after recrystallization from ethyl acetate-ligroin (Found : C, 65.44 ; H, 6.63. Calcd. for $C_{19}H_{24}O_6$, C, 65.50 ; H, 7.28).

Delactonization of the C-2 keto-dihydro-G A_1 (IX).

The C-2 keto derivative (250 mg) and 25% sulfuric acid (30 ml) were boiled for 2 hrs. on an oil bath (135°), in a current of nitrogen and evolved gas was collected in a Barium hydroxide solution. The evolution of about one mole of carbon dioxide was estimated by back-titration of the Barium hydroxide solution. Then, the reaction mixture was extracted with ethyl acetate and treated in the usual way to be separated into acidic and neutral fractions. The acidic product (125 mg) was purified by partition chromatography of silicic acid using 1 M phosphate buffer solution of pH. 5.2 as the stationary phase and benzene-*n*-buthanol (97 : 3 v/v) as the mobile phase. The product (X) was obtained as prisms, d.p. 215~217° after recrystallization from ethyl acetate-ligroin (Found : C, 70.15 ; H, 8.16. Calcd. for $C_{18}H_{24}O_4$, C, 71.02 ; H, 7.95).

The neutral fraction (XI) (60 mg) was obtained in crystalline form and showed m.p. 193° after recrystallization from ethyl acetate-ligroin (Found : C, 78.20 ; H, 8.90. Calcd. for $C_{17}H_{24}O_2$, C, 78.42 ; H, 9.29).

Reductoin of the methyl ester of deoxo-product (V) with Lithium aluminum hydride.

Lithium aluminum hydride (500 mg) was added to the solution of the methyl ester (650 mg) in tetrahydrofuran (100 ml) and boiled for 20 hrs.. Then the excess of reagent was decomposed with moistened ethyl acetate, the precipitate thus formed being filtered off, and the organic layer was evaporated. The product (XII) was obtained as short prisms, m.p. 214~219° after recrystallization from tetrahydrofuran-ethyl acetate (Found : C, 64.90 ; H, 9.20. Calcd. for $C_{18}H_{32}O_5$, C, 65.8 ; H, 9.70).

Dehydrogenation of the pentaol (XII) with selenium.

a) The pentaol (XII) (450 mg) was mixed with selenium powder, and heated for 2 hrs. at 360° followed by 380~400° for 0.5 hr.. The reaction mixture was extracted with benzene and the extracts were distilled by 1 mm/Hg at 140~150°. The distillate (110 mg) was dissolved in light petroleum (40~60°) and chromatographed through a column of alumina (Brockmann, 0.5 × 10 cm), obtaining a crystal, m.p. 54~67°. After recrystallization five times from dil. ethanol, the m.p. was raised up to 94~97° and was shown to be identical with 1,3-dimethyl fluorene (XIII) as mentioned above.

b) A mixture of (XII) and selenium powder (1.0 g)

was heated at 340~360° for 2.5 hrs.. The reaction mixture was extracted with benzene and the extract was distilled by 1 mm/Hg at 150~180°. The distillate was dissolved in light petroleum (40~60°) chromatographed through a column of alumina (Brockmann, 0.5×10 cm) and eluted with the same solvent, and a hyrocarbon was obtained which was considered to be a mixture of 1,3,9-trimethyl fluorene and 1,9-dimethyl fluorene as mentioned above. Then, the column was eluted with light petroleum-ether (8 : 2 v/v), obtaining a fluorenol (27 mg) as long prisms. m.p. 152~154° after recrystallization from ligroin (Found: C, 85.45; H, 6.87. Calcd. for $C_{15}H_{14}O$, C, 85.7; H, 6.6 for $C_{14}H_{12}O$, C, 85.7; H, 6.1).

Acknowledgement The authors are indebted to Kyowa Fermentation Industries Ltd., Takeda Pharmaceutical Co., Ltd., Merck & Co., Inc., and Pfizer & Co., Inc. for kindly supplying us the crystalline gibberellins, and to Mr. K. Aizawa of Department of Agricultural Chemistry, University of Tokyo for infrared measurements.

This study was supported in part by the Grant in Aid for Agricultural Research from the Ministry of Education, to which the authors wish to express their gratitude.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 6, p. 509~524, 1959]

Biochemical Studies on "Bakanae" Fungus. Part 53.
Chemical Structure of Gibberellins. Part XIX.

By Nobutaka Takahashi, Yasuo Seta, Hiroshi Kitamura and Yusuke Sumiki

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Received December 17, 1958

The outline of this report has already been published in this Journal as Communication to the Editors[1,2]. It was shown that gibberellin A_3 (gibberellic acid) has one more double bond at certain position of the gibberellin A_1 structure. Several experiments supporting the partial structure of gibberellin A_3 as (I) are reported. The position of the double bond in ring A of gibberellin A_3 was assigned and the final structure of gibberellin A_3 is reported.

In the previous reports[3,4], it was shown that gibberellin A_3 (gibberellic acid) has the same functional groups as that of gibberellin A_1, i.e. one secondary hydroxyl, one tertiary hydroxyl and one lactone group, except a double bond. This result suggests that the hydrogenation of gibberellin A_1 and A_3 may give the same compound. From this point of view, we attempted the catalytic reduction of gibberellin A_1 and A_3 in order to clarify the structural relation between these gibberellins.

When gibberellin A_3 methyl ester was hydrogenated with Adams' catalyst, the acidic fraction in 80% yield, and the neutral fraction in 15~20% yield were obtained on uptaking of 1.8 moles of hydrogen. The acidic fraction was a mixture consisting of two kinds of crystals which could be separated by partition chromatography of silicic acid. One was formulated as $C_{20}H_{30}O_6$, d.p. 212~6°, and the other as $C_{20}H_{30}O_6$, d.p. 224°.

1) N. Takahashi, Y. Seta, H. Kitamura and Y. Sumiki, This Bulletin, **21**, 327 (1957).
2) N. Takahashi, Y. Seta, H. Kitamura and Y. Sumiki, *ibid.*, **22**, 432 (1958).
3) H. Kitamura, Y. Seta, N. Takahashi, A. Kawarada and Y. Sumiki, *ibid.*, **21**, 71 (1957).
4) H. Kitamura, Y. Seta, N. Takahashi, A. Kawarada and Y. Sumiki, *ibid.*, **23**, 408 (1959).

Recrystallization of the neutral fraction gave a crystal of melting point 235~8° which was identical with dihydrogibberellin A_1 methyl ester (a mixture of two stereoisomers) in all respects, melting point and infrared spectrum. When free gibberellin A_3 was subjected to the hydrogenation with Adams' catalyst, it took up two moles of hydrogen and the following four kinds of crystals which could be separated by the partition chromatgraphy of silicic acid treated with pH 5.2 phosphate buffer were obtained. Recrystallization of the fraction obtained from 8% butanol-benzene effluent gave a monobasic acid, $C_{19}H_{26}O_6$ d.p. 270~2°, which was identical with dihydrogibberellin A_1 in all respects, melting point and infrared spectrum. By recrystallization of the mother liquor a small quantity of crystal of d.p. 268~270°, $C_{19}H_{24}O_6$, was obtained. The infrared spectrum of this crystal was quite similar to that of gibberellin A_1 but it was not identical. From this fact, we supposed that this substance might be a stereoisomer of gibberellin A_1 which resulted from reduction of one of the double bonds of gibberellin A_3. The other hydrogenation products were two kinds of dicarboxylic acids. From the effluent of 15% butanol-benzene, a crystal of d.p. 290~5°, $C_{19}H_{28}O_6$, was obtained in 20% yield. From the effluent of ethanol, the fourth crystal was obtained, d.p. 184~6° and the formular, $C_{19}H_{28}O_6 \cdot 3/2H_2O$, was assigned to this crystal. Methylation of dibasic acid of d.p. 290~5° with diazomethane gave dimethyl ester of m.p. 204°. It was identical with dimethyl ester which was obtained by methylation of crystal of d.p. 224°, hydrogenolysis product of gibberellin A_3 methyl ester. But, the dimethyl ester (m.p. 130~2°) of dibasic acid of d.p. 184° was not identical with dimethyl ester (m.p. 140°) obtained by methylation of another crystal of d.p. 212~6°, hydrogenolysis product of gibberellin A_3 methyl ester.

The fact that dihydrogibberellin A_1 was obtained in a fairly good yield from hydrogenation products led us to conclude that gibberellin A_3 has one more double bond in some position of the gibberellin A_1 structure. The fact that the acidic fraction was obtained from neutral ester and dicarboxylic acid from free acid on the catalytic hydrogenation indicates that hydrogenolysis of the lactone ring happened and also that one of the double bonds of gibberellin A_3 and lactone alkyl oxygen are located in the allylic position each other. Validity of our above conclusion is shown by the following experiment. When gibberellin A_1 and A_3 methyl esters were treated with an excess of lithium aluminum hydride in boiling tetrahydrofuran for 24 hours, $C_{19}H_{32}O_5$, m.p. 208~210°, from gibberellin A_1 methyl ester (A_1 Li) and $C_{19}H_{30}O_5$, m.p. 208~10°, from A_3 methyl ester (A_3 Li) were obtained. A_1 Li gave tetraacetyl derivative, $C_{27}H_{40}O_9$, m.p. 148°, by treatment of sodium acetate anhydride at 130° and a triacetyl derivative, $C_{25}H_{38}O_8$, m.p. 198~200°, by treatment of acetic anhydride in pyridine at room temperature. A_3 Li gave a triacetyl derivative, $C_{25}H_{36}O_8$, m.p. 182~3° by treatment of pyridine and acetic anhydride at room temperature. Tetraacetyl derivative of A_3 Li was syrup and could not be purified into crystal. As tetraacetyl A_1 Li shows a OH absorption band in its infrared spectrum, it is clear that these reduction products are pentaols and further, the lactone alkyl oxygen is probably tertiary because that tertiary OH of gibberellin A_1 in rings C and D is acetylable under this acetylation condition. On hydrogenation with Adams' catalyst, A_1 Li absorbed no hydrogen and the original substance was recovered. A_3 Li absorbed 1.6 moles of hydrogen, yielding a crystal in a 60% yield which is identical with A_1Li. It is unusual that the exocyclic methylene of gibberellin A_1 was reduced in the course of lithium aluminum hydride reduction as shown in the above hydrogenation experiment. Therefore, the ozonolysis reaction was attempted. As ozonolysis of A_1 Li, tri- and tetra acetyl A_1 Li in acetic acid gave formaldehyde in a very poor yield which was identified as formdimedone it was most probable that exocyclic methylene does not remain in A_1 Li.

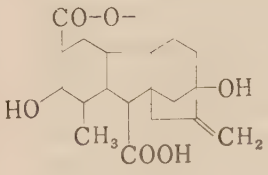
As the close similarity of structure of gib-

berellin A_1 and A_3 was thus confirmed, elucidation of the chemical structure of gibberellin A_3 can be conducted in connection with that of gibberellin A_1. Especially, in the case of gibberellin A_3, it is convenient to carry out experiments, taking advantage of the existence of the double bond in ring A. The following experiments of hydrogenolysis products offer the data which is necessary to derive the partial structure of gibberellin A_1 and A_3 as (I), reported in the previous paper[5,6].

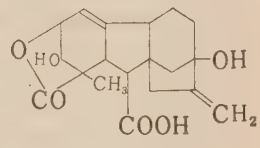
Methylation of dibasic acid of d.p. 290~5° by the Fischer-Speier method gave acidic monomethyl ester, $C_{20}H_{30}O_6$, m.p. 258~60°, which was not identical with a crystal of d.p. 224°, hydrogenolysis product of gibberellin A_3 methyl ester. This fact shows that a carboxyl group derived from lactone group by hydrogenolysis was esterified. As a carboxyl group which attach to tertiary carbon can not be esterified by the Fischer-Speier method, the lactone group was considered to be attached to a secondary carbon. Dibasic acid of d.p. 290°, was oxidized with anhydrous chromic acid in acetic acid at 60° and reaction products were purified through silicic acid chromatography. Crystal of d.p., 198~200°, $C_{18}H_{26}O_4$ was obtained in a relatively low yield. This compound was shown to be monobasic by titration. This result shows that the secondary hydroxyl group was oxidized and simultaneous decarboxylation occurred on grounds of the β-keto acid system. The above two results can be interpreted by the partial structure of (I).

When dibasic acid of d.p. 290~5° was boiled in acetic anhydride, d.p. 165~7°, $C_{23}H_{30}O_7$, was obtained in which two hydroxyl groups were acetylated. In the same way, dibasic acid of m.p. 185° gave the anhydride, d.p. 186~8°, $C_{23}H_{30}O_7$. Their infrared spectra show typical anhydride bands at 1800 cm^{-1} and 1762 cm^{-1}, and anhydride formation between two carboxyl groups at 3 and 9 positions was posible in only one stereochemical configuration.

Although Cross et al. proposed the formula (II) as gibberellin A_3[7,8] it is clear that it is invalid because the attaching position of lactone carbonyl was proved to be 3 by the lithium aluminum hydride experiment as show in the former report[5,6]. Also, the followieg series of experiments on periodic acid oxidation shows that Cross' structure is incorrect.



(I)



(II)

A_1 Li and A_3 Li do not consume periodate in either alkaline or an acidic condition for 24 hours. These results clearly indicate that lactone oxygen is not located at the vicinal position to the original secondary hydroxyl group in ring A. In order to confirm this fact, we attempted the periodic acid oxidation to the substance obtained by alkali treatment in which the lactone ring was hydrolysed.

On alkali treatment of gibberellin A_1, dihydrogibberellin A_1, isogibberellin A_1 and gibberellin A_3, each of them consumed two moles of alkali. Then, the solution back-titrated to pH 8~9 was treated with potassium dimesoperiodate solution at the same pH. In this experiment, dihydrogibberellin A_1 and isogibberellin A_1,—the latter is an isomer obtained by treatment of gibberellin C, do not consume periodate for a period of 24 hours. However, gibberellin A_1 consumed about one mole of periodate rather slowly while gibberellin A_3 consumed more than 4 moles of periodate very rapidly. We, therefore, examined changes caused by alkali treatment under the same condition

5) Y. Seta, N. Takahashi, H. Kitamura, and Y. Sumiki, This Bulletin, **22**, 429 (1958).
6) Y. Seta, N. Takahashi, A. Kawarada, H. Kitamura and Y. Sumiki, *ibid.*, **23**, 412 (1959).
7) B. E. Cross, J. F. Grove, J. MacMillan and T. C. Mulholland, *Chem. and Ind.*, **36**, 954 (1956).
8) B. E. Cross, J. F. Grove, J. MacMillan and T. C. Mulholland, *Proceedings of Chem. Soc.* **August of 1958**, 221.

as described above, excluding periodic oxidation. The reaction product recovered by acidifying the solution treated by alkali was chromatographed on buffered silicic acid. Gibberellin A_1 in a 10% yield and an isomer of gibberellin A_1 (A_1 alkali isomer) in a 40% yield and syrup in a 20% yield were obtained. The latter is a different compound from pseudogibberellin A_1 and does not consume periodate under the above alkaline condition. In connection with the fact that dihydrogibberellin A_1 and isogibberellin A_1 do not consume periodate, it might be considered that a small and not the main portion of the alkali treated product, which cannot be isolated in a pure state through partition chromatography of the silica gel, consumed periodate.

While the behavior of gibberellin A_3 to alkali is different from those of other compounds, the silicic acid chromatography of alkali treatment product gave two kinds of substances. One was obtained from the effluent of 10% buthanol-benzene and could not be purified in the crysatalline form (F-1) and the other was obtained from 20% buthanol-benzene effluent in a white amorphous form, $C_{19}H_{26}O_8$, d.p. 154° (F-2). As the latter compound is dibasic acid, the lactone opening of gibberellic acid is irreversible. In acidic and alkaline conditions, F-2 consumes periodate very rapidly and F-1 consumes periodate rather slowly only in alkaline condition. Moreover, F-2 absorbs one mole of hydrogen by catalytic hydrogenation. The elementary analytical datum shows that two moles of water are added to the original formula, one of them being used in hydrolysis of the lactone ring. The result of catalytic reduction shows that one of the two double bonds present in gibberellin A_3 is absent in amorphous dibasic acid. Consequently, we concluded that dibasic acid is not a simple hydrolysis product of the lactone ring but a secondarily changed product—perhaps being due to hydration of the double bond—and thus, the newly introduced hydroxyl group is vicinal to the lactone alkyl oxygen. The results of periodic acid oxidation are summarized in Tables I, II and III.

TABLE I. THE CONSUMPTION OF PERIODATE BY A_1 Li and A_3 Li

	hr.	2	6	12	24
A_1 Li	acidic	0.1 mol			0.2 mol
	alkaline	0 //			0 //
A_3 Li	acidic	0.2 //	0.3 mol	0.4 mol	0.5 //
	alkaline	0 //			0 //

TABLE II. THE CONSUMPTION OF PERIODATE BY ALKALI-TREATED SOLUTION (back-titrated)

hr.	2	24
Gibberellin A_1 ester	0.5 mol	0.8 mol
Dihydrogibberellin A_1	0 //	0 //
Isogibberellin A_1	0 //	0 //
A_1 alkali isomer	0 //	0 //
Gibberellin A_3 ester	3.5 //	>4.0 //

Table III. THE CONSUMPTION OF PERIODATE BY ALKALI DEGRADATION PRODUCTS OF GIBBERELLIN A_3 METHYL ESTER

	hr	2	24
F-1	acidic	0.1 mol	0.25 mol
	alkaline	0.35 //	1.1 //
F-2	acidic	0.8 //	0.9 //
	alkaline	3.5 //	>4.0 //

Although in our former report[5,6], we proposed the formula of gibberellin A_1 as (V), alkyl oxygen of lactone group was assigned by somewhat ambiguous evidences. It is desirable to ascertain the location of the double bond of gibberellin A_3 in ring A_1, independently from gibberellin A_1 and to assign the position of lactone alkyl oxygen in connection with the double bond.

When gibberellin A_3 was treated with bromine in tetrahydrofuran at $-5\sim0°$, monobromogibberellin A_3, $C_{19}H_{21}O_6$ Br, d.p. $240\sim2°$, in a 40% yield and another crystal, $C_{19}H_{21}O_6Br$, d.p. $268\sim270°$, in a low yield were separated. The former monobromogibberellin A_3 gave a monoacetyl derivative, $C_{21}H_{23}O_7Br\cdot H_2O$, m.p. 175° by pyridine-acetic anhydride at room temperature. Methyl ester of this monoacetyl derivative, $C_{22}H_{25}O_7$ Br, m.p. $178\sim180°$ was obtained by treatment with diazomethane. This substance shows no hydroxyl absorption band. Mono-

bromogibberellin A_3 shows a maximum peak at 290 mμ (ε=30) in the ultraviolet spectrum. In the course of conversion from gibberellin A_3 to monobromo derivative, a hydroxyl group was converted to a carbonyl group. The close similarity of this change of the functional group in bromination of gibberellin A_3 to the case of gibberellin A_1[9,10] led us to conclude that the Wagner rearrangement of rings C, D of gibberellin A_3 occurred. This conclusion was supported by the fact that monobromogibberellin A_3 gave gibberellin C on debromination with zinc and acetic acid accompanying simultaneous reduction. It was shown by the catalytic hydrogenation of monobromogibberellin A_3 methyl ester that the double bond other than exocyclic methylene in gibberellin A_3 remains unaffected at the same position as that of gibberellin A_3. This is because on hydrogenation of monobromogibberellin A_3 methyl ester by Adams' catalyst in methanol, the neutral fraction in a 40% yield and acidic fraction in a 60% yield were obtained. From the neutral fraction, monobromogibberellin A_1 methyl ester d.p. 264~6°, was obtained. The acidic fraction, hydrogenolysis product of the lactone ring, gave a crystal $C_{20}H_{25}O_6Br$, d.p. 206° by silicic acid chromatography. Thus, monobromogibberellin A_3 can be used to determine the position of the double bond in ring A without being affected by the double bond in ring D, i.e. the exocyclic methylene.

When monoacetyl monobromogibberellin A_3 methyl ester was treated with ozone in chloroform solution and ozonide was decomposed by steam distillation, a nonvolatile acidic substance was obtained. Although the ozonolysis product could not be isolated into the crystalline form by various methods, the result of titration of white powder obtained by silicic acid chromatography showed that this product is monobasic. This result led us to conclude that double bond exists at C–4~C–11. Because, if the double bond exists at the position of C–1~C–10 or C–10~C–11, the oxidation product by ozone should not be carboxylic acid but a diketo compound. The position of C–1~C–2, C–2~C–3 and C–3~C–4 are excluded by the fact that the hydroxyl of gibberellin A_3 is not enol and also that gibberellin A_3 is neither α,β-unsaturated acid nor lactone.

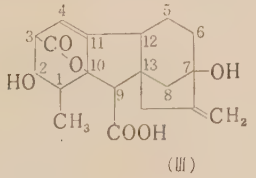
Moreover, when the ozonolysis product was boiled with 33% H_2SO_4, evolution of 0.5 mole of carbon dioxide was observed. This easiness of decarboxylation shows that the monobasic acid is β-oxocarboxylic acid. From these facts, we assigned the position of the double bond of monobromogibberellin A_3 and gibberellin A_3 to the same C–4~C–11. It has also been clarified that the alkyl oxgen of lactone is allylic to the double bond, because of easy occurrence of hydrogenolysis on the catalytic hydrogenation of gibberellin A_3 and monobromogibberellin A_3.

If we put the double bond at C–4~C–11, the alkyl oxgen of the lactone ring should be attached to C–12 or C–10. From the fact[7] that aromatization of gibberellin A_3 occurs with great ease, this position C–12 seems unlikely; As a result, the structure of gibberellin A_3 is formulated as (III) and monobromogibberellin A_3 as (IV) respectively and gibberellin A_1 as (V) such as reported in the previous paper[5].

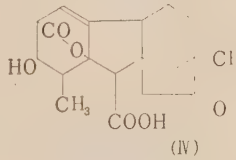
As shown in (I), (III), (IV) and (V), the lactone of gibberellins is δ and not γ. In the infrared spectrum of gibberellin A_1 and A_3, the lactone

9) N. Takahashi, Y. Seta, H. Kitamura and Y. Sumiki, This Bulletin, **21**, 75 (1957).

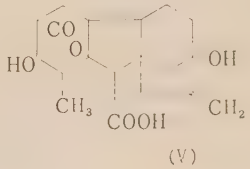
10) N. Takahashi, Y. Seta, H. Kitamura, A. Kawarada and Y. Sumiki, *ibid.*, **23**, 493 (1959).



(III)



(IV)



(V)

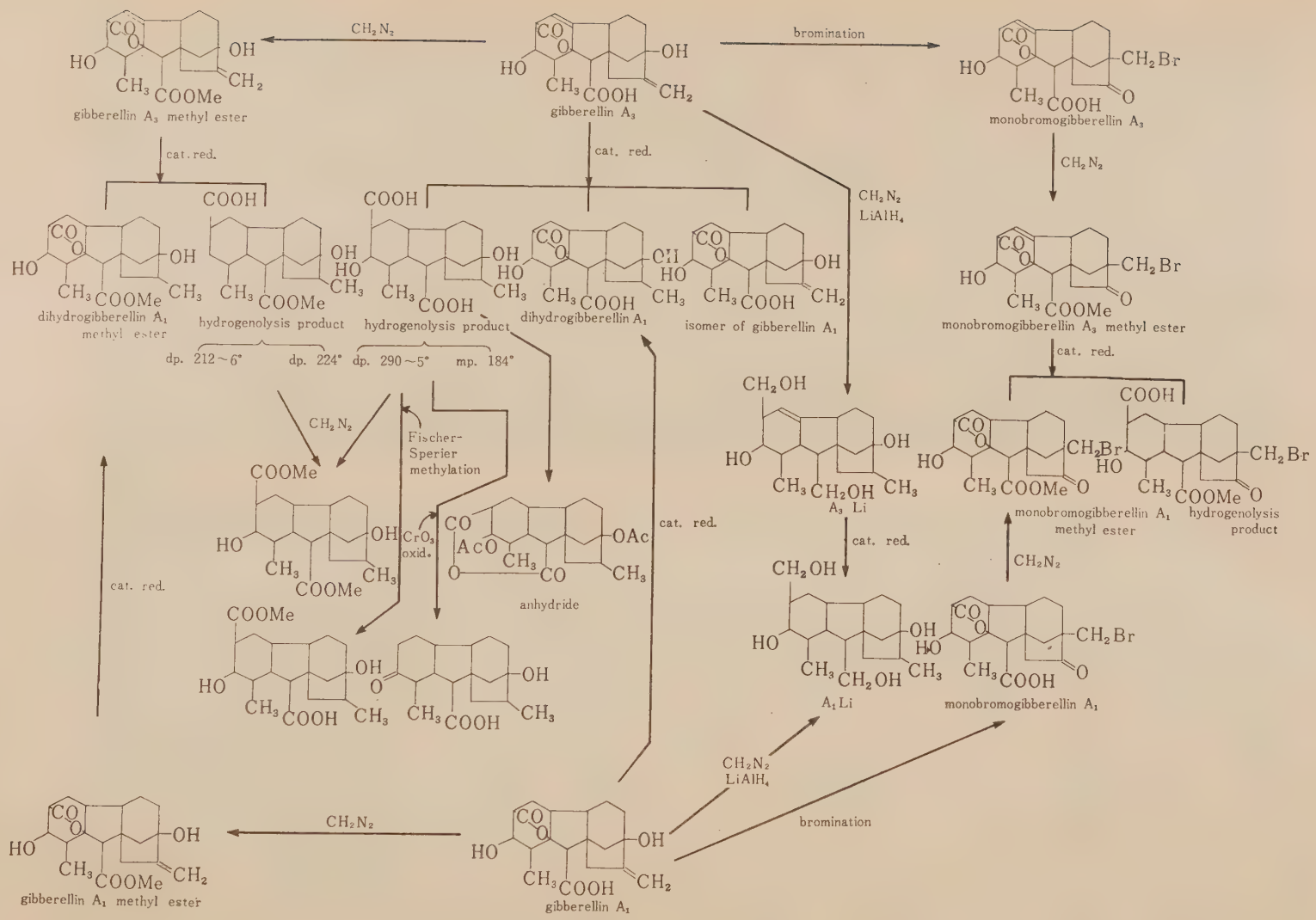
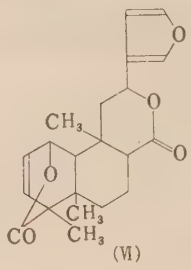
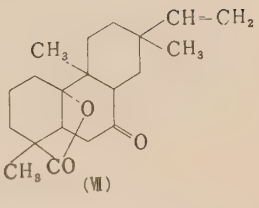


FIG. 1. Relation between Gibberellin A_1 and Gibberellin A_3

carbonyl band of A_1 methyl esters appears at 1759 cm^{-1} and that of A_3 methyl ester at 1766 cm^{-1} which are usually thought to be γ-lactone. But, if a lactone ring does exist in the ring system, there might be a shift of the carbonyl band to a high freqency for the structural strain.

In fact, the carbonyl band of δ-lactone of columbin (VI) appears at 1750 cm^{-1} and that of γ-lactone of rosenonolactone (VII) at 1786 cm^{-1}.[3]





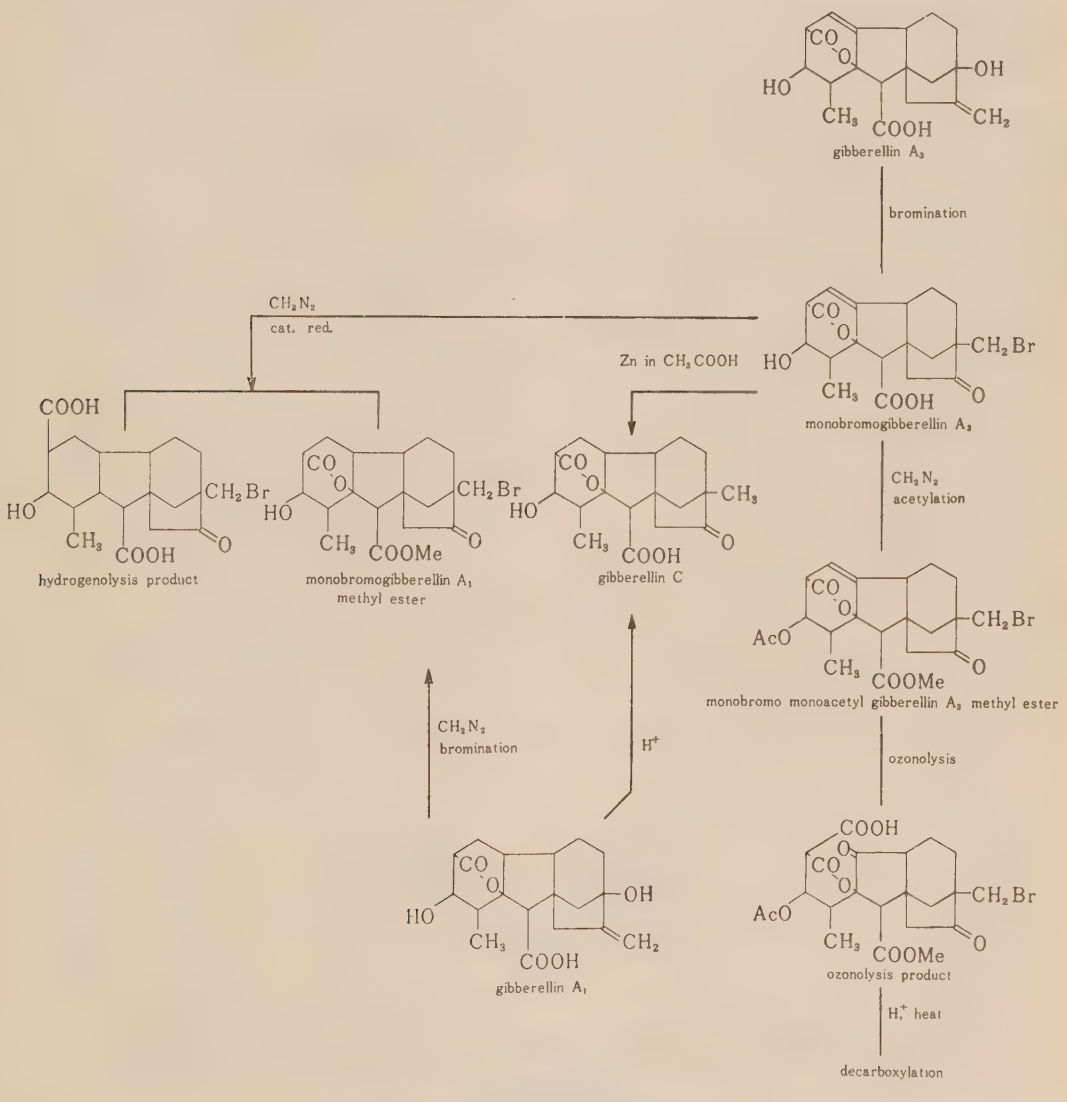


FIG. 2. Degradation of Gibberellin A_3

From these examples, it is reasonable to consider that carbonyl absorption band of gibberellins show the existence of δ-lactone.

The relation between gibberellin A_1 and gibberellin A_3, and degradation process of gibberellin A_3 are summarized in Figs. 1 and 2.

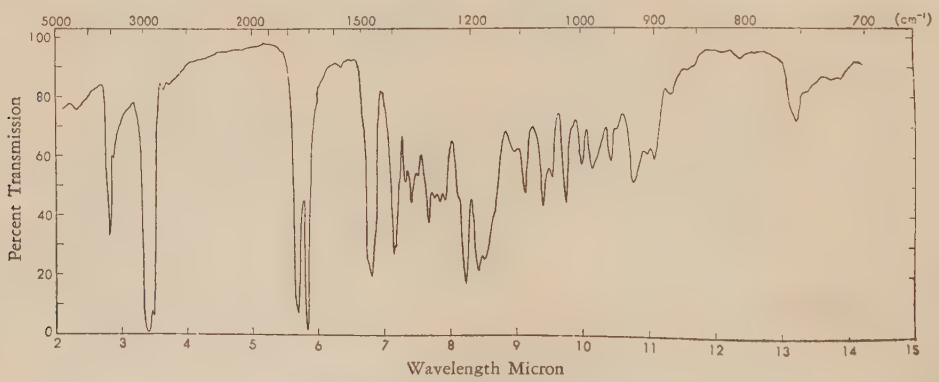
EXPERIMENTAL

1) Hydrogenation of gibberellin A_3

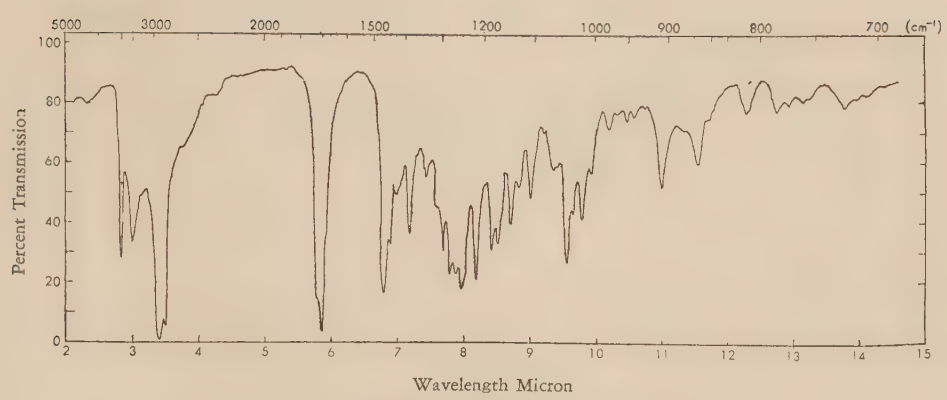
a) Gibberellin A_3 methyl ester: Three hundred mg of gibberellin A_3 methyl ester was hydrogenated over 70 mg of Adams' catalyst in methanol and 1.8 moles of hydrogen were absorbed for thirty minutes. On evaporation of the solvent, the residual matter was dissolved in ethyl acetate and extracted with aq. $NaHCO_3$. Recrystallization of the neutral fraction (45 mg) gave a rod, m.p. 235~8° which was identical with dihydrogibberellin A_1 methyl ester in both respects of melting point and infrared spectrum.

The acidic fraction (240 mg) was subjected to silicic acid chromatography. Twenty five g of silicic acid was mixed with 18 ml of pH 5.4 phosphate buffer and packed with a solvent of 4% buthanol-benzene in column. The sample was dissolved in a solvent having the same composition and then subjected to chromatography. Elution was continued with same solvent composition. The volume of each fraction was 18 ml. From fractions 4~5, 130 mg of syrup was obtained and was crystallized into rods, d.p. 212~6° from ethyl acetate and ligroin. Found: C, 65.94; H, 7.80; Calcd. for $C_{20}H_{30}O_6$; C, 65.55; H, 8.25; By methylation with diazomethane, dimethylester was obtained, m.p., 130~2°. Found: C, 65.98; H, 7.72; Calcd. for $C_{21}H_{32}O_6$: C, 66.30; H, 8.47; Recrystallization of fractions 7~11 (60 mg) gave a crystal of d.p. 224°. Found: C, 64.75; H, 8.20; Calcd. for $C_{20}H_{30}O_6$: C, 65.55; H, 8.25; Esterification of this crystal with diazomethane gave a crystal of m.p. 204~5°. Found: C, 66.02; H, 8.67; Calcd. for $C_{21}H_{32}O_6$: C, 66.30: H, 8.47.

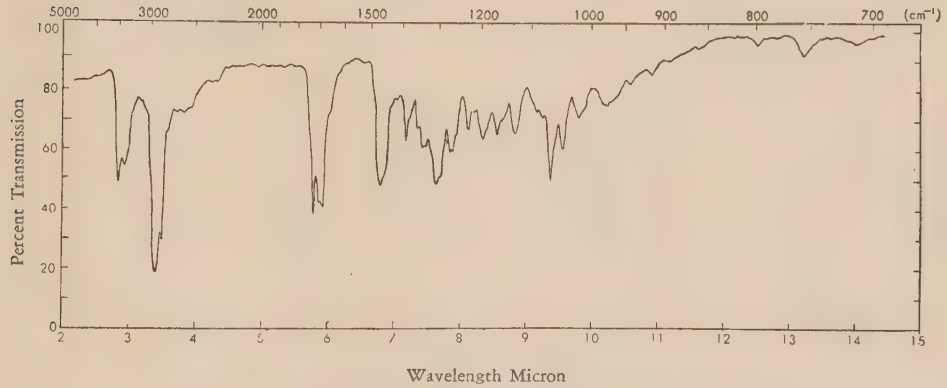
b) Gibberellin A_3: Five hundred mg of gibberellin A_3 was hydrogenated over 85 mg of Adams' catalyst in methanol and 2.3 moles of hydrogen were absorbed for one hour. The residual matter obtained on evaporation of the solvent was separated into four components by silicic acid chromatography and recrystallization. Forty g of silicic acid was treated with 30 ml of pH 5.2 phosphate buffer. As the eluting solvent, 8% buthanolbenzene was used. The volume of each fraction was 20 ml. Fractions 11~15 gave 40 mg of crystal, d.p. 178~180°. Fractions 18~40 gave 215 mg of crystal. This fraction was recrystallized from diluted ethanol into rod d.p. 270~2° which was identical with dihydrogibberellin A_1 in all respects. Recrystallization of the mother liquor from ethyl acetate and ligroin gave a small quantity of crystal, d.p. 268~270°, infrared spectrum of which was quite similar to that of gibberellin A_1 but not identical. Its methyl ester, m.p. 242~4°, was different from gibberellin A_1 methyl ester ester in both respects of melting point and infrared spectrum. This crystal was considered to be a stereoisomer of gibberellin A_1 resulting from the reduction of one double bond of gibberellic acid. At this stage the solvent composition was changed to 15% buthanolbenzene. This elution of 500 ml gave 100 mg of another crystal, d.p. 290~5°. Found: C, 64.71; H, 8.19; Calcd. for $C_{19}H_{28}O_6$: C, 64.75; H, 8.01; It was determined by titration that this crystal was dibasic acid. Methylation of this crystal by diazomethane gave



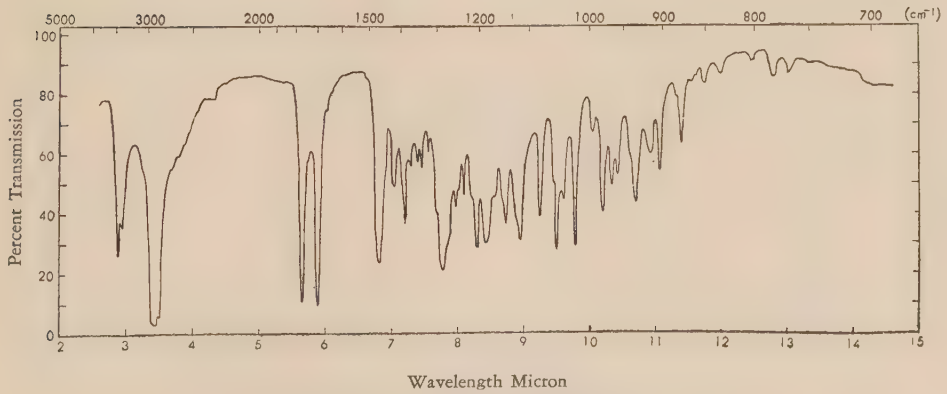
I.R.—(1) dihydrogibberllin A_1 methyl ester



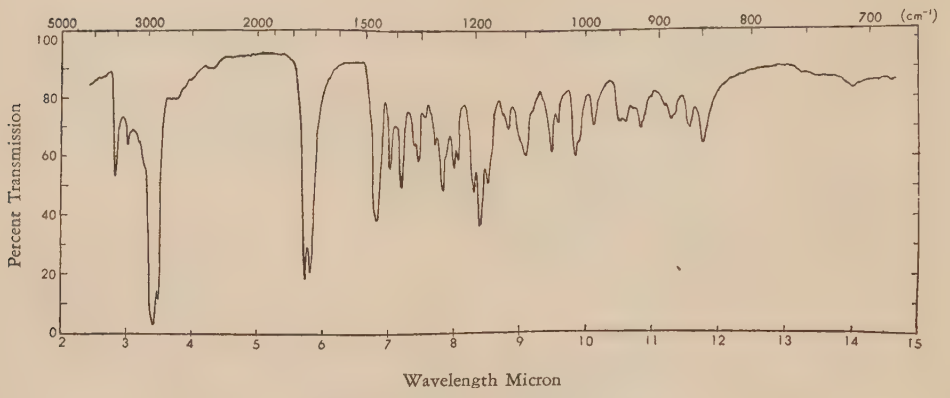
I.R.—(2) A_3 methyl ester hydrogenolysis product m.p. 212~4°



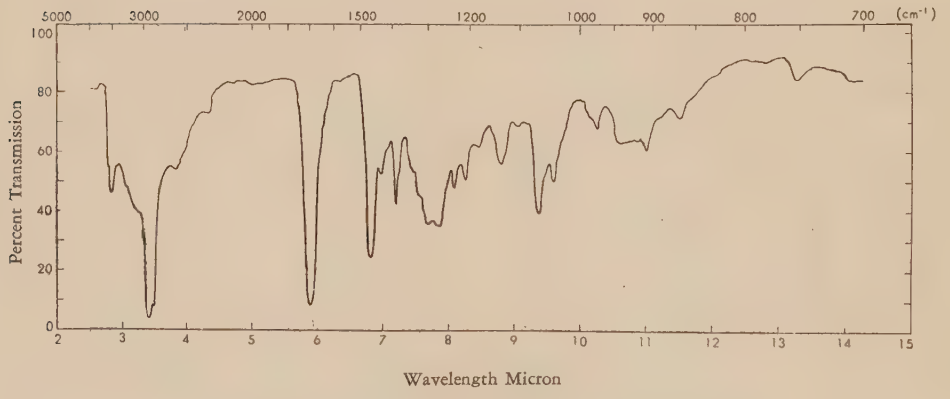
I.R.—(3) A_3 methyl ester hydrogenolysis product m.p. 224°



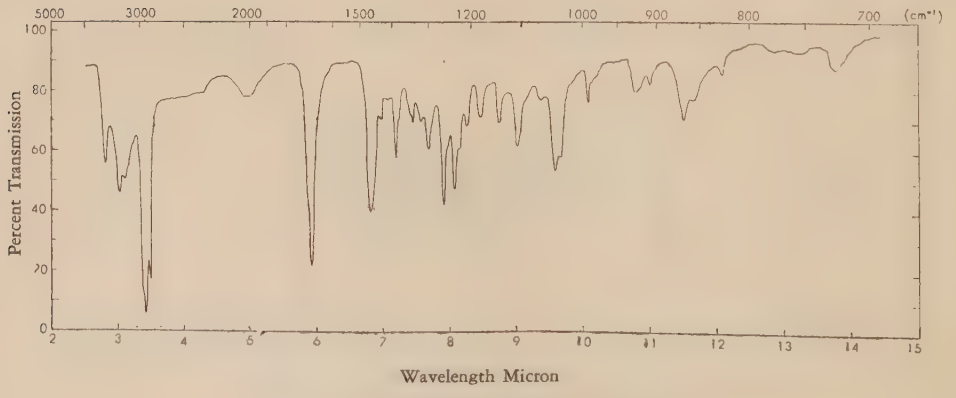
I.R.—(4) dihydrogibberellin A_1



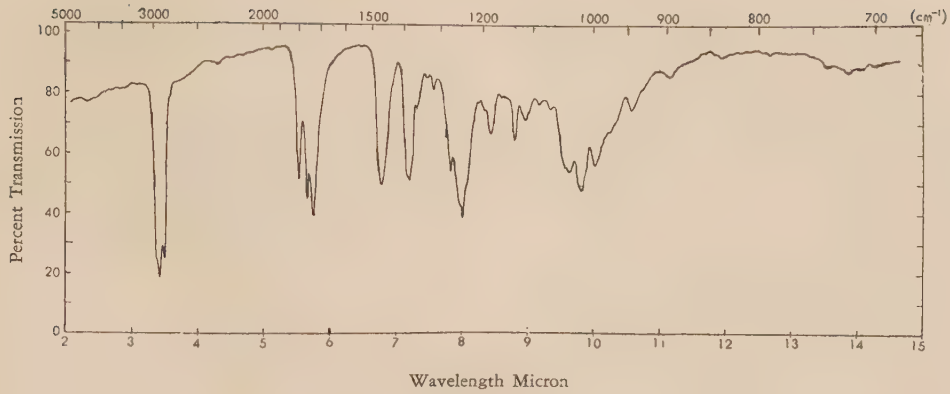
I.R.—(5) isomer of gibberellin A_1



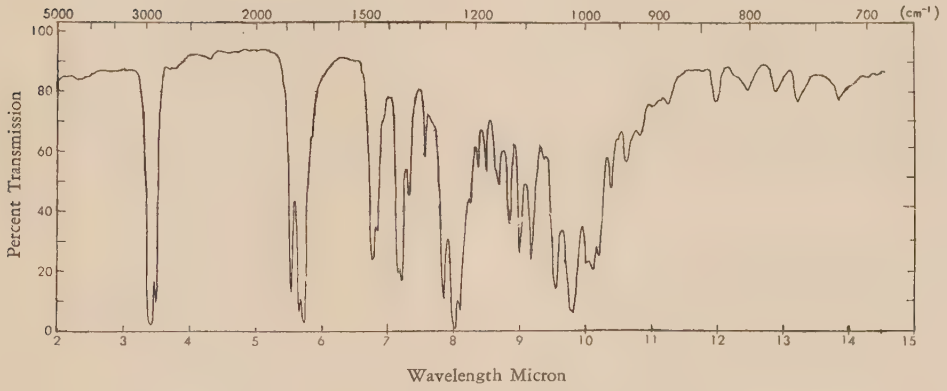
I.R.—(6) hydrogenolysis product d.p. 290~5°



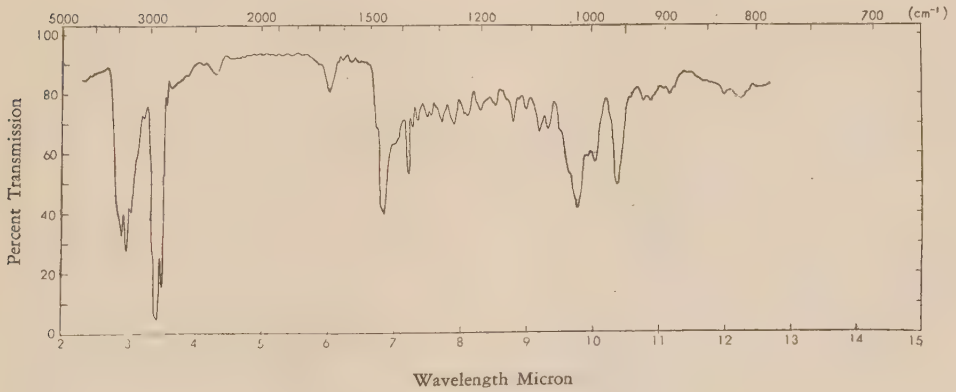
I.R.—(7) hydrogenolysis product m.p. 184~6°



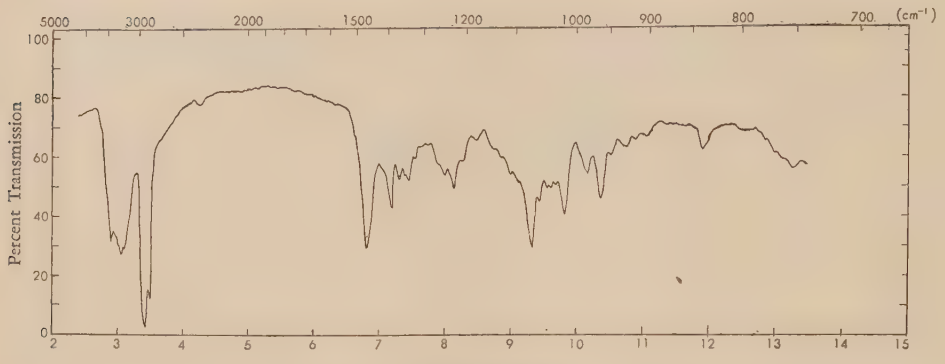
I.R.—(8) anhydride d.p. 165~8°



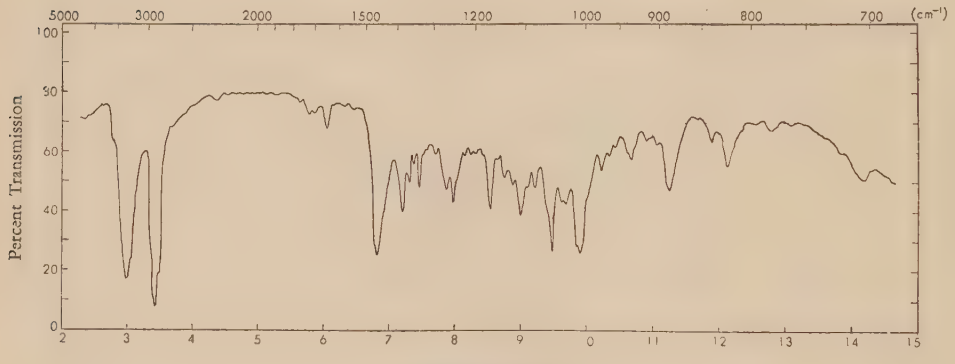
I.R.—(9) anhydride d.p. 185~6°



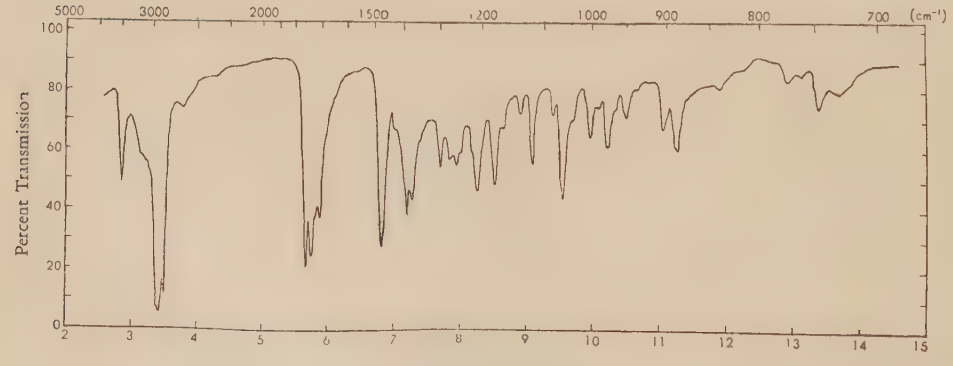
I.R.—(10) A_1 Li (hydrate)



I.R.—(11) A_1 Li



I.R.—(12) A_3 Li



I.R.—(13) monobromogibberellin A_3

a dimethyl ester of m.p. 204°. Found: C, 66.18; H, 8.50; OCH_3, 15.64, Calcd. for $C_{21}H_{32}O_6$: C, 66.30; H, 8.47; OCH_3, 16.32.

The column was washed with 300 ml of ethanol and ethanol was evaporated and water added to the residual matter. Needles precipitated, d.p. 184~6.° Infrared spectrum of this crystal and that of crystal obtained from fractions 5~7 were identical. Found: C, 60.92; H, 7.72; Calcd. for $C_{19}H_{28}O_6$ $3/2H_2O$: C, 60.48; H, 7.45; Dried over P_2O_5 at 100° Found: C, 63.67; H, 7.82; Calcd. for $C_{19}H_{28}O_6 \cdot 1/2H_2O$: C, 63.51; H, 7.52; This crystal was determined by titration as dibasic acid.

Methylation of this crystal by diazomethane gave a dimethyl ester of m.p. 140°. Found: C, 66.96; H, 7.89; OCH_3, 16.03; Calcd. for $C_{21}H_{32}O_6$: C, 66.30; H, 8.48; OCH_3, 16.32.

c) The Fischer-Speier methylation of dibasic acid, d.p. 290~5°: One hundered and fifty mg of crystal of d.p. 290~5° was dissolved in 5 ml of methanol and 0.4 ml of con. H_2SO_4 was added to this solution. The solution was boiled for 30 minutes. The reaction mixture was diluted with water and extracted with ethyl acetate. The acidic fraction (155 mg) was chromatographed on silicic acid (15 g) treated with pH 5.2 phosphate buffer (11 ml) with 4% buthanol-benzene. The effluent was cut by each 15 ml. Fractions 12~25 gave a crystal m.p. 258~260° (50 mg). Found: C, 65.28; H, 8.53; OCH_3, 8.45; Calcd. for $C_{20}H_{30}O_6$: C, 65.55; H, 8.25; OCH_3, 8.47.

d) Oxidation of dibasic acid, d.p. 290~5° by anhydrous chromic acid: Three hundred mg of dibasic acid, d.p. 290~5°, was dissolved in 10 ml of acetic acid and 117 mg of CrO_3 in 10 ml of acetic acid was added to this sample solution for 30 minutes at 60°. After warming for one hour at 60°, the acetic acid was distilled off. The residual matter was extracted with ether continuously. The acidic fraction (220 mg) was chromatographed on silicic acid (silicic acid 20 g, pH 5.2 phosphate buffer 15 ml, 3% buthanol-benzene) and each 15 ml of effluent was collected as one fraction. Fractions 8~15 gave 79 mg of crystal, d.p. 198~200°, recrystallized into rods.
Found: C, 69.59; H, 8.57; Calcd. for $C_{18}H_{26}O_4$: C, 70.59; H, 8.49.

e) Anhydride of dibasic acid, d.p. 290~5°: One hundred and fifty mg of dibasic of d.p. 290~5° was heated in acetic anhydride at 130~140° for 2 hours. Acetic anhydride was distilled off in vacuo. The residual syrup was crystallized from ethyl acetate-ligroin into a crystal of d.p. 165~8° (40 mg).
Found: C, 65.85; H, 6.83; Calcd. for $C_{23}H_{30}O_7$: C, 66.01; H, 7.23.

f) Anhydride of dibasic acid, d.p. 184~6°: One hundred and fifty mg of dibasic acid of m.p. 184~6° was treated in the same manner as that of d.p. 290~5°. Crystal of d.p. 185.6° (50 mg) was obtained. Found: C, 65.86; H, 6.56. Calcd. for $C_{23}H_{30}O_7$: C, 66.01; H, 7.23.

2) Reduction of Lithium aluminum hydride

a) Gibberellin A_1 methyl ester: Three hundred mg of gibberellin A_1 methyl ester was boiled with 100 mg of $LiAlH_4$ in tetrahydrofuran for 18 hours. The reaction mixture was treated with ethyl acetate and a small quantity of water. The precipitation was separated by centrifuge and washed with tetrahydrofuran several times. The combined solution was evaporated and ethyl acetate was added to the residual matter under warming. Fine needles precipitated, m.p., 140~2°. This crystal contained one mole of crystallization water.
Found: C, 63.91; H, 8.78; Calcd. for $C_{19}H_{32}O_5 \cdot H_2O$: C, 63.66; H, 9.56; Recrystallization from hot ethyl acetate gave an anhydrous crystal, m.p. 204°. Found: C, 66.98; H, 9.16. Calcd. for $C_{19}H_{32}O_5$: C, 67.03; H, 9.47.

Tetraacetyl A_1 Li Three hundred mg of A_1 Li was warmed with sodium acetate in acetic anhydride at 150° for 2 hours. After cooling, ethanol was added and the solvent was evaporated. The residue was dissolved in ethyl acetate and washed with aq. $NaHCO_3$ The ethyl acetate layer was evaporated. Recrystallization from ethyl acetate-ligroin gave 80 mg of crystal, m.p. 148~150°. Found: C, 63.71; H, 7.20; Calcd. for $C_{27}H_{40}O_9$: C, 63.76; H, 7.93; The result of acetyl value determination showed that this compound is tetraacetyl derivative.

Triacetyl A_1 Li Two hundred mg of A_1 Li was treated with acetic anhydride in pyridine at room temperature for 48 hours. Pyridine was evaporated in vacuo and the residual matter was treated with water and extracted with ethyl acetate. On evaporation of the solvent and recrystallization, a crystal of m.p. 198~200° (130 mg) was obtained. Found: C, 64.45; H, 8.23; Calcd. for $C_{25}H_{36}O_8$: C, 64.36; H, 8.21; The number of acetyl radical was found to be three, after determination by the Kuhn-Roth method.

b) Gibberellin A_3 methyl ester: The reduction was performed following the same procedure as in the case of gibberellin A_1 methyl ester. Recrystallization from

hot ethyl acetate gave a crystal of m.p., 208~210°, Found: C, 67.41; H, 8.36; Calcd. for $C_{19}H_{30}O_5$: C, 67.43; H, 8.94.

Triacetyl A_3 Li Triacetyl A_3 Li was obtained in the same way as that of A_1 Li, m.p., 182~3°. Fouud: C, 64.43; H, 7.39; Calcd. for $C_{25}H_{36}O_8$: C, 64.63; H, 7.81.

3) Hydrogenation of A_1 Li and A_3 Li

a) A_1 Li: One hundred and fifty mg of anhydrous A_1 Li was subjected to the hydrogenation with 75 mg of Adams' catalyst. For one hour, 0.3 mole hydrogen was absorbed but the original substance was recovered on evaporation of the solvent.

b) A_3 Li: One hundred and fifty mg of A_3 Li was hydrogenated over Adams' catalyst and 1.5 mole of hydrogen were uptaken. The crystal of m.p. 205~8° (100 mg) was obtained on recrystallization from ethyl acetate. This substance was identical with anhydrous A_1 Li in both respects of melting point and infrared spectrum.

4) Alkali treatment of gibberellin A_1 and A_3

a) Gibberellin A_1 methyl ester: Three hundred mg of gibberellin A_1 methyl ester was refluxed in 12 ml of 1/2 N NaOH for 2 hours. The reaction mixture was extracted with ethyl acetate and next acidified with dil H_2SO_4 and reextracted with ethyl acetate. The acidic fraction (300 mg) was chromatographed on silicic acid (20 g) treated with pH 5.2 (17 ml) phosphate buffer with a solvent of 10% buthanol-benzene. Each 18 ml of the effluent was collected as one fraction. Fractions 7~9 gave 34 mg of crystal, m.p. 244~5° which was identical with gibberellin A_1 in the respect of infrared spectrum. Fractions 12~30 gave 165 mg crystal (A alkali isomer) which showed a double melting point at 154° and 216~8°. It contained one mole of crystallization water and the water was lost by drying over P_2O_5 at 100° in vacuo. Hydrate, Found: C, 62.42; H, 7.08; Calcd. for $C_{19}H_{24}O_6 \cdot H_2O$: C, 62.28; H, 7.15; Anhydrous compound; Found: C, 65.18; H, 7.18; Calcd. for $C_{19}H_{24}O_6$: C, 65.50; H, 6.94. The column was eluted with 20% buthanol-benzene. This fraction gave 50 mg of syrup which could not be purified in the crystalline form.

b) Gibberellin A_3 methyl ester: Three hundred mg of gibberellin A_3 methyl ester was boiled with 0.1N NaOH for 30 minutes. Two hundred and seventy mg of the acidic fraction extracted with ethyl acetate was subjected to silicic acid chromatography. Thirty g of silicic acid treated with 22 ml of pH 5.2 phosphate buffer was packed into a column with 10% buthanol-benzene. Sixty mg of syrup (F-1) was obtained from effluent of 10% buthanol-benzene. Elution with 20% buthanol-benzene gave 200 mg of syrup. Recrystallization from ethyl acetate-ligroin gave amorphous powder, (F-2) d.p. 152~4°. Found: C, 59.82; H, 7.30; Calcd. for $C_{19}H_{26}O_8$: C, 59.67; H, 6.85. Hydrogenation of F-2 over Adams' catalyst absorbed one mole of hydrogen.

5) Periodic acid oxidation

a) Oxidation in acidic condition: Twenty five mg of the sample was dissolved in one ml of ethanol and 1 ml of periodic acid (0.3 M) was added. The reaction mixture was filled up to 10 ml. Four ml of solution was pipetted and the residual periodate in each 4 ml of solution was determined by the following procedure. Ten ml of sat. $NaHCO_3$, 4.0 ml of 0.05 M Na_2AsO_3 solution saturated with $NaHCO_3$ and 1 ml of KI were added and titrated with I (0.05 M).

b) Oxidation in alkaline condition: Instead of 1 ml of periodic acid, 2 ml of potassium dimesoperiodate solution was used. Potassium dimesoperiodate solution was prepared by neutralizing the periodic acid with KOH to pH 8~9. A_1 Li, A_3 Li, F-1 and F-2—alkali treated products of gibberellin A_3—were oxidized under the above-two conditions. In oxidation of F-1 and F-2 in alkaline condition, the sample was neutralized before the addition of oxidation solution. These results are summerized in Tables I and III.

c) Twenty five mg of gibberellin A_1 methyl ester, dihydrogibberellin A_1 and isogibberellin A_1, A_1 alkali isomer and gibberellin A_3 methyl ester were boiled in 0.5 N NaOH for 2 hours. The boiling time for gibberellin A_3 was 30 minutes and 0.1 N NaOH was used. The reaction mixture was back-titrated to pH 8~9 with 0.1 N NaOH. A potassium dimesoperiodate solution was added to this solution and filled up to 10 ml. The consumption of periodate of each 4.0 ml was determined after 2 hours and 24 hours, respectively. The results are summarized in Table II.

6) Bromination of gibberellin A_3

Four hundred mg of gibberellin A_3 in 10 ml of tetrahydrofuran was cooled by an ice salt mixture at −5~0°. Six ml of bromine-tetrahydrofuran solution (700 mg of bromine in 10 ml of tetrahydrofuran) was added to this sample solution for one hour under stirring. After additional stirring for one hour, tetra-

hydrofuran was distilled off in vacuo. The residual syrup was dissolved in ethyl acetate and extracted with aq. $NaHCO_3$. The acidified $NaHCO_3$ layer was extracted with ethyl acetate. On evaporation of the solvent, the residual syrup (480 mg) was obtained which was subjected to silicic acid chromatography (silicic acid 30 g, pH 5.4 phosphate buffer 20 ml, 3% buthanol-benzene). Each 18 ml of the effluent was collected as one fraction. Fractions 7~15 gave 160 mg of syrup which was recrystallized into rods, d.p. 242~4°. Found: C, 53.85; H, 5.12; Br, 19.87; Calcd. for $C_{19}H_{21}O_6Br$: C, 53.65; H, 4.94; Br, 18.59; Its methyl ester was obtained by treatment with diazomethane, recrystallized into crystal of m.p. 214~6° by ethyl acetate-ethanol-ligroin. Found: C, 55.32; H, 5.32; Br, 17.56; Calcd. for $C_{20}H_{23}O_6Br$: C, 54.65; H, 5.24; Br, 18.22.

Monoacetyl monobromogibberellin A_3 was obtained by treatment of pyridine-acetic anhydride at room temperature for 48 hours, recrystallized by ethanol-ethyl acetate-ligroin, m.p. 175°. Found: C, 52.34; H, 5.92; Br, 18.02; Calcd. for $C_{21}H_{23}O_7Br \cdot H_2O$: C, 51.96; Br, 16.50.

Monoacetyl monobromogibberellin A_3 methyl ester was obtained when the acetyl derivative was treated with excess of diazomethane, recrystallized into needles, m.p. 178~180°. Found: C, 55.15; H, 5.58; Br, 16.64; Calcd. for $C_{22}H_{25}O_7Br$: C, 54.89; H, 5.20; Br, 16.64.

From the mother liquor of recrystallization of monobromogibberellin A_3, another crystal, d.p. 268~270°, was obtained in fine needles. Found: C, 53.85; H, 4.96; Br, 19.30; Calcd. for $C_{19}H_{21}O_6Br$: C, 53.65; H, 4.94; Br, 18.59.

Its methyl ester was obtained by treatment with an excess of diazomethane recrystallized into fine needles by ethyl acetate-ligroin, m.p. 202~4°.
Found: C, 55.24; H, 5.68; Br, 17.45; Calcd. for $C_{20}H_{23}O_6Br$: C, 54.65; H, 5.23; Br, 18.22.

7) Catalytic hydrogenation of monobromogibberellin A_3 methyl ester

Three hundred mg of monobromogibberellin A_3 methyl ester was subjected to catalytic hydrogenation using Adams' catalyst in methanol. About one mole of hydrogen was uptaken. On evaporation of the solvent, the residual matter was dissolved in ethyl acetate and separated into neutral and acidic fractions in the usual manner. The neutral fraction (115 mg) gave a crystal, of d.p. 264~6° by recrystallization of ethanol which was identical with monobromogibberellin A_1 methyl ester. The acidic fraction (180 mg) was subjected to the silicic acid chromatography (silicic acid 17 g, pH 5.2 phosphate buffer 11 ml, 3% buthanol-benzene). The first eluted fraction of 70 ml gave 50 mg of crystal, d.p. 206°. Found: C, 53.75; H, 5.95; Br, 17.75; Calcd. for $C_{20}H_{27}O_6Br$: C, 54.18; H, 6.09; Br, 18.06.

8) Debromination of monobromogibberellin A_3

Three hundred mg of monobromogibberellin A_3 in 15 ml of acetic acid was refluxed with 2 g of zinc for 4 hours. After the reaction mixture was cooled, zinc and zinc acetate were filtered off. On evaporation of acetic acid, 230 mg of residual syrup was obtained and was subjected to the silicic acid chromatography (silicic acid 25 g, pH 5.4 phosphate 18 ml, 3% buthanol-benzene). Seventeen ml of effluent was collected as one fraction. Fractions 2~6 gave 75 mg of syrup which could not be purified in the crystalline form. Fractions 11~20 gave 100 mg of crystal, d.p. 245°, which was identical with gibberellin C in all respects (m.p. and infrared spectrum).

9) Ozonolysis of monoacetyl monobromogibberellin A_3 methyl ester

Four hundred mg of monoacetyl monobromogibberellin A_3 methyl ester was dissolved in 40 ml of chloroform. Ozone enriched oxygen was passed through this sample solution under cooling with dry ice and methanol mixture for 2 hours. After evaporation of the solvent at room temperature in vacuo, the residual syrup was steam distilled. Volatile carbonyl compound was not obtained. The non-volatile fraction was extracted with ethyl acetate. The acidic fraction (380 mg) was chromatographed on silicic acid (silicic acid 40 g, pH 5.4 phosphate buffer 30ml, 3% buthanol-benzene). Five percent-buthanol-benzene effluent gave 150 mg of syrup which could not be crystallized but a white powder was obtained by drying in vacuo. The titration of this powder showed it to be monobasic. Found: C, 48.79; H, 5.14; Br, 13.76; Calcd. for $C_{22}H_{25}O_{10}Br$: C, 49.91; H, 4.72; Br, 15.12.

10) Decarboxylation of ozonolysis product

Fifty mg of ozonolysis product was boiled with 10 ml of 1 : 3 H_2SO_4 at 140° for 2 hours. Carbon dioxide was trapped by $Ba(OH)_2$ solution using a nitrogen gas flow. Barium hydroxide solution was backtitrated with 0.1 N H_2SO_4. Evolution of 0.5 mole of carbon dioxide was observed.

Acknowledgement We are indebted to Kyowa Fermentation Co., Merck & Co., Inc., and Chas. Pfizer & Co. Inc. for supplying gibberellin A_3 used in this work. Thanks are also due to Mr. Aizawa for infrared spectra, Mrs. Sato, Miss Suzuki, Miss Isobe of our Department for microanalyses. This research was supported in part by the Grant in Aid for Agricultural research defrayed by the Ministry of Education.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 6, p. 524~527, 1959]

Studies on the Reduction of Terpenes with Sodium in Aqueous-ammonia

Part III. On the Reduction (−)-Carvone*

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Received March 6, 1959

On the reduction of (−)-carvone with sodium in aqueous-ammonia, the predominant product was found to be (−)-dihydrocarveol, a new stereoisomer. From this fact, it might be concluded that this reduction method is stereospecific for (−)-carvone, similary as in the case of (−)-menthone. By the catalytic hydrogenation of (−)-dihydrocarveol, a new stereoisomer of carvomenthol has also been prepared. It is noteworthy that (−)-dihydrocarveol has the same conformation (e, e, e) as that of (−)-menthol, which was also quantitatively obtained from (−)-menthone by application of our method of reduction reported previously.

On reduction, carvone (I) affordes theoretically three ketones, dihydrocarvone (II), carvotanacetone (III), and carvomenthone (IV); and four alcohols, carveol (V), dihydrocarveol (VI), carvotanacetol (VII), and carvomenthol (VIII). These are shown in Fig. 1. On the catalytic hydrogenation of I, it was possible to obtain either III, IV or VIII[1,2,3,4]. By the use of aluminum isopropoxide, V was produced[5]; and, with sodium and alcohol VI was yielded[5], while with zinc dust in sodium hydroxide or acetic acid solution II was prepared[6].

In the previous papers of this series, it has been shown that (−)-menthone was reduced quantitatively to (−)-menthol by our method using sodium in aqueous-ammonia (Part I)[7], and moreover, inverted menthone to (+)-isomenthol in good yield (Part II)[8].

In this investigation, (−)-carvone was treated with sodium in aqueous-ammonia according to the same procedures as that described previously[7].

* For Part II see this Bulletin, **23**, 380 (1959).

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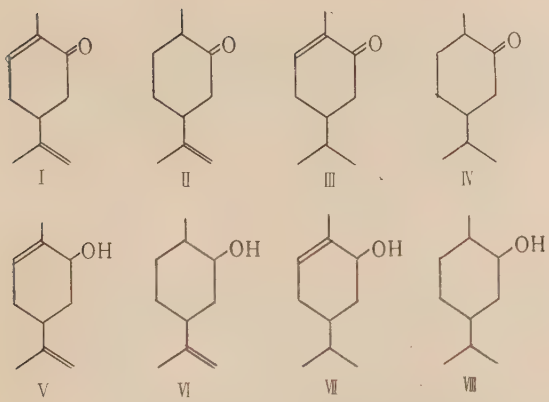

FIG. 1. Carvone and its Reduction Products

The reduction product was shown to be composed of (1) alcohols (90%), (2) carbonyl compounds (7.5%), and (3) unreduced carvone (2.5%).

(1) From the alcohol fraction, 3,5-dinitrobenzoate, m.p. 121.5~122.5°, was separated in good yield (82.2%). It was confirmed to be (−)-dihydrocarveol from elementary analysis of 3,5-dinitrobenzoate and infra-red spectrum of regenerated alcohol. The others seemed to be a mixture of isomeric dihydrocarveol, and carveol was not identified.

(2) Semicarbazone, m.p. 191~192°, isolated from carbonyl fraction, did not depress the melting point on admixture with semicarbazone of (+)-dihydrocarvone which was prepared by chromic acid oxidation of (−)-dihydrocarveol. It is possible to consider that (+)-dihydrocarvone is an intermediate between (−)-carvone and (−)-dihydrocarveol.

On the basis of these data, it seemed reasonable to assume that a preferential attack was made against the ketone group and double bond conjugated with ketone in carvone, but not against ethylene of the isopropenyl group.

On reduction, (−)-carvone theoretically should give rise to four optically active dihydrocarveols, as indicated in Fig. 2.

The predominant production of (−)-dihydrocarveol might conclude that this reduction method is stereospecific for (−)-carvone, whereas, Read[5] obtained (+)-dihydrocarveol from (+)-carvone in about 50% yield with sodium and alcohol. It is interesting that (−)-dihydrocarveol and (−)-menthol[7], obtained from natural ketones by this reduction method, have the same conformation (e,e,e).

In this experiment, the double bond of (−)-dihydrocarveyl-3,5-dinitrobenzoate was determined by bromine-method, using pyridine-bromine-sulphate[9]; and (−)-dihydro-dibromocarveyl-3,5-dinitrobenzoate, m.p. 153~153.5°, was obtained from the titrated solution. It was shown that the bromine-method can be applied to determine the double bond of unsaturated alcohol nitrobenzoates, while the catalytic hydrogenation method was found to be unsuitable since the nitro group was attacked.

The infra-red spectra of (−)-dihydrocarveol and (−)-carvomenthol are shown in Fig. 3. The spectrum of the former showed a strong band at 885 cm^{-1}, and its C=C stretching frequency appeared at 1638 cm^{-1}, which closely agreed with 889, 1641 cm^{-1} of isopulegol, reported by Macbeth[10].

Both (−)-dihydrocarveol, (+)-dihydrocarvone and (−)-carvomenthol are new isomers, while the dextrotatory series were reported by Read[11].

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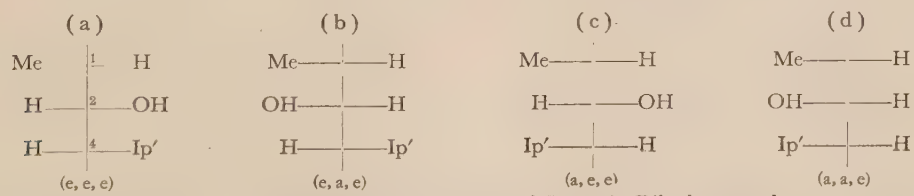

FIG. 2. Stereochemical Relationships of Isomeric Dihydrocarveols
(a) dihydrocarveol, (b) neodihydrocarveol, (c) isodihydrocarveol, (d) neoisodihydrocarveol; (Ip′ = C_3H_5)

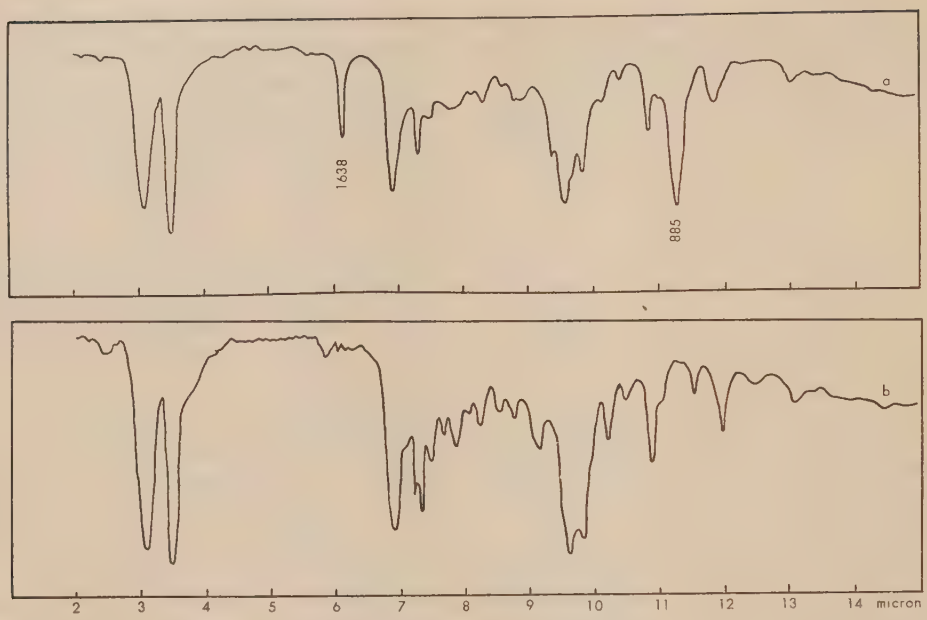

FIG. 3. Infra-red Absorption Spectra of (−)-Dihydrocarveol (a) and (−)-Carvomenthol (b). (in oil)

EXPERIMENTAL

All melting and boiling points were uncorrected. Ultra-violet absorption spectra were determined in methanol employing a Beckman Model DU-quarz spectrophotometer. Infra-red spectra were recorded in oil using a Shimadzu double beam spectrophotometer at Prof. Nakajima's Laboratory, University of Kyoto. Microanalyses were carried out by the Microanalytical Division, Prof. Mitsui's Laboratory, University of Kyoto.

(−)-Carvone

From Japanese spearmint oil (140 g), (−)-carvone (75 g) was separated by treatment with sodium sulfite; b.p. 85~86°/5 mm, d_{24}^{24} 0.9578, n_D^{24} 1.4970, $[\alpha]_D^{30}$ −62.94° (homog.), MR 45.89 (calcd. 45.26), λ_{max} 235 mμ, E_{max} 9170, iodine-number 322.76 (calcd. 337.98); The carbonyl content was 91.44% by the hydroxylamine-method. oxime m.p. 72~73°, semicarbazone m.p. 141°, 2,4-dinitrophenylhydrazone m.p. 188.5~189.5°.

Reduction of (−)-carvone

(−)-Carvone (10.0 g) was reduced with sodium (7.0 g) in aqueous-ammonia (20 ml) and benzene (20 ml), according to the procedure described previously[7]. The reduction temperature was kept between 3° and 10° under cooling with ice and salt. The product (7.5 g) was obtained by the usual method; b.p. 114.7~116.5°/23 mm, d_{24}^{24} 0.9253, n_D^{24} 1.4756, $[\alpha]_D^{33}$ −23.13° (homog.), λ_{max} 235 mμ, E_{max} 226 (carvone content 2.5%), iodine-number 191.97; The carbonyl content was 10.12% by the hydroxylamine-method. These values showed that one of two double bonds of carvone was saturated.

Separation of carbonyl compound

To a solution of semicarbazide-hydrochloride (0.7 g), sodium acetate anhydride (0.5 g) and water (1.5 ml), was the reduction product (6.0 g) added. To this mixture was added methanol dropwisely till it became clear. After being allowed to stand one night, methanol was removed under diminished pressure and the residue was extracted with ether. When the ether extract was washed with water, semicarbazone (Semi-I) crystallized. After removal of ether from this filtrate, the residue was distilled to obtain alcohol fraction (4.7 g), while semicarbazone (Semi-II) was remained in the distillation residue.

(−)-Dihydrocarveyl-3,5-dinitrobenzoate

The alcohol fraction obtained by the treatment described above had the following properties; b.p. 89~90°/5 mm, d_{24}^{24} 0.9213, n_D^{24} 1.4768, MR 47.30 (calcd. 47.24 for dihydrocarveol), $[\alpha]_D^{24}$ −26.70° (homog.), iodine-number 188.65.

Esterification of this alcohol fraction (3.793 g) with 3,5-dinitrobenzoylchloride (8.5 g) and pyridine (20 ml)

at room temperature for 24 hrs. gave a crude ester, m.p. 102～115°, (8.275 g, Yield 96.6%), from which was isolated by one crystallization from methanol pure (−)-dihydrocarveyl-3,5-dinitrobenzoate as colourless needles, m.p. 121.5～122.5°, $[\alpha]_D^{20}-52.12°$ (c 15.926 in chloroform), iodine-number 72.30 (calcd. 72.86), (6.803 g, Yield 82.2% from crude ester). *Anal.* Found: C, 58.62; H, 6.06. Calcd. for $C_{17}H_{20}O_6N_2$: C, 58.61; H, 5.79%.

It was an optical enantimorphs of (+)-dihydrocarveyl-3,5-dinitrobenzoate prepared by Read.

Crystallization of the material, m.p. 85～95°, (0.626g), recovered from the mother liquors from the purification of (−)dihydrocarveyl-3,5-dinitrobenzoate, gave a product of m.p. 92～96°, iodine-number 74.23, (0.451 g). From the determination of iodine-number, the ester of lower melting point probably consisted of mixture of isomeric dihydrocarveyl-3,5-dinitrobenzoate, however, could not be purified satisfactorily by recrystallization.

(−)-Dihydrocarveol

(−)-Dihydrocarveyl-3,5-dinitrobenzoate (6.5 g) was hydrolysed by refluxing for 4.5 hrs. with N/2-alcoholic potassium hydroxide (90 ml). Pure (−)-dihydrocarveol (2.60 g) was prepared by distillation under diminished pressure, as a colourless oil. Yield 90.2%; b.p. 107～107.2°/14 mm, d_{20}^{23}0.9202, n_D^{23}1.4748, MR 47.17 (calcd. 47.24), $[\alpha]_D^{23}-33.25°$ (homog). Its infra-red spectrum is shown in Fig. 3.

(−)-Carvomenthol

(−)-Dihydrocarveol (1.456 g) was dissolved in glacial acetic acid (10 ml), and was hydrogenated by using of platinum oxide (30 mg) as a catalyst. It absorbed 223.5 ml (17°) of hydrogen (99.5% for theor.), and gave (−)-carvomenthol (1.209 g, Yield 82.0%); b.p. 99.8～100.3°/12 mm, d_{17}^{17}0.9034, n_D^{17}1.4633, MR 47.67 (calcd. 47.62), $[\alpha]_D^{17}-25.12°$ (c 17.12 in benzene). Its infra-red spectrum is listed in Fig. 3.

(−)-Carvomenthol gave the corresponding 3,5-dinitrobenzoate as colourless needles, m.p. 106～107°; *Anal.* Found: C, 58.32; H, 6.04. Calcd. for $C_{17}H_{22}O_6N_2$: C, 58.28; H, 6.33%.

(+)-Dihydrocarvone-semicarbazone

(−)-Dihydrocarveol (703 mg) was oxidized by a Beckman's chromic acid at 50°. After removal of ether from the obtained ether extract, (+)-dihydrocarvone-semicarbazone was obtained by the usual method, Crude semicarbazone (685 mg) showed m.p. 185～186°, recrystallizations from methanol raised the m.p. to 191～192° (560 mg). *Anal.* Found: C, 63.31; H, 9.11. Calcd. for $C_{11}H_{19}ON_3$: C, 63.13; H, 9.15%.

Identification of Semi-I and Semi-II

(a) Semi-I (420 mg) was a colourless needles, m.p. 178～180°. One crystallization from methanol brought the m.p. up to 191～192°; it remained constant after further recrystallization. $[\alpha]_D^{20}-28.06°$ (c 3.706 in chloroform-methanol).

(b) Semi-II (0.8 g) was semi-crystalline. After repeating recrystallizations from methanol, a pure semicarbazone, m.p. 191～192°, (129 mg) was obtained.

Both semicarbazones (total 442 mg) were identical with the above mentioned (+)-dihydrocarvone-semicarbazone.

Acknowlegement The authors are indebted to Prof. S. Takei, Prof. T. Mitsui, Prof. M. Nakajima and Assoc. Prof. T. Hashizume of the University of Kyoto for their kind advices and suggestions. They also wish to express their sincere thanks to Prof. T. Mitsui for elementary analysis, Prof. M. Nakajima for infra-red spectra, and to Mr. K. Fujimoto, Miss. H. Utsunomiya and Miss. T. Uemura for assistance in carrying out this experiment.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 6, p. 528~532, 1959]

Influence of Smoking Procedures on Combustion Temperature of Cigarettes and the Nicotine Content of Cigarette Smoke*

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Received March 12, 1959

In order to establish fundamental knowledge on the combustion mechanism of tobacco, the effects of smoking procedures on both combustion temperature of cigarettes and the amount of nicotine transferred into cigarette smoke were investigated. The combustion temperatures measured with fine thermocouples specially devised, and an excellent responsive autorecording potentiometer, were 794~827°C, irrespective of smoking procedures. The free burning temperature obtained was 746°C.

When the cigarette was smoked up to a definite length (45 mm) from the lighted end, the amount of nicotine in cigarette smoke increased with increment in puff velocity, showing almost a linear curve.

Recently, numerous investigations have been conducted on the measurement of combustion temperatures of cigarettes, cigars and pipe tobaccos by many workers, from the physiological viewpoint.

Wynder[1], using an iron-constantan thermocouple, reported the combustion temperature of cigarette to be 682°C. Seelkopf[2] found the average temperature to be 742°C with a platinum and platinum-rhodium thermocouple under the conditions of puff duration of 2.5 sec. Lam[3], using a 0.2 mm. diameter iron-constantan thermocouple, reported that the temperatures of cigarettes, cigarillos and cigars were 866°C, 822°C, and 813°C, respectively. Greene[4], using 28-gauge chromel-alumel thermocouples, found that the temperatures of cigarettes (610~740°C), of cigars (580~660°C) and of pipes (540~590°C). Harlow[5], using No. 40 B & S gauge platinum and platinum-rhodium thermocouples, measured the temperature as 774°C under the conditions of 35 ml/sec. puff velocity and a two second-puff duration. He also found the free-burning temperature to be 746°C. Ermala[6] reported that the temperatures of cigarettes, cigars and pipes were 470~812°C, 380~620°C and 380~620°C, respectively. Touey[7], very recently, indicated that most of the reported temperatures were obtained under poorly defined smoking conditions; and, his study was carried out in an air-conditioned room, and the cigarettes used were selected on the basis of moisture content, air flow resistance and weight. The combustion temperatures reported by Touey were in the range of 873~890°C, regardless of smoking conditions.

Nicotine is the most important tobacco smoke constituent, and, with other related alkaloids, its behavior accompanied with smoking has long been a subject of wide interest. An appreciable amount of works in concern of their identification[8], determination[9,10,11], and pyrolysis of nicotine[12] have been reported in the literature.

* Studies on the thermal decomposition of tobacco alkoloids. Part I.

1) E. L. Wynder, *Cancer Res.*, **13**, 855-864 (1953).
2) C. Seelkopf, *Z. Lebensm. -Untersuch. u. -Forsch.*, **100**, 218-222 (1955).
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In previous studies[13,14] conducted on cigarette smoke, it has been demonstrated that the amount of nicotine in cigarette smoke exceedingly increases accompanied with increment of the smoked length of cigarette.

The work reported here is concerned with the effects of smoking procedures on both combustion temperature and the amount of nicotine transferred into the main-stream, using domestic commercial regular-size cigarettes which were selected on the basis of moisture content and weight.

EXPERIMENTAL

Preparation of Sample and Smoking Conditions The cigarettes used were of 70 mm-length and blended with domestic flue cured, Indian flue cured, domestic naturally cured and Indian naturally cured tobaccos. After being conditioned to 10.93% moisture content with a relative humidity of 58% at 20°C, the cigarettes falling in the weight range of 0.960 g ±13 mg.

The smoking conditions were as follows: Puff velocities—5 ml/sec, 10 ml/sec, 20 ml/sec, 40 ml/sec and 60 ml/sec. Puff duration—two seconds, twice per minute. In all experiments, the cigarettes were smoked up to a definite length (45 mm) from the lighted end. Smoking experiments were carried out in a room maintained at relative humidity of 59.7%±1.6% and at 19.4±1°C.

Cigarette Smoking Procedure A diagram of the apparatus used for smoking is shown in Fig. 1. The cigarette was inserted into the tube-end of the three-way stop cock C, and covered air-tightly with a short strip of rubber tube, and the cigarette end connected with the rubber tube was sealed with Duco cement to avoid leakage of air. Suction for the puff was created by a vacuum pump G which was connected with a flowmeter F, and puff velocities were adjusted by this flowmeter. The puffing action was accomplished by turning the three-way stop cock C by hand. A cotton plug approximately 5 g in weight and impregnated with 2 ml of 5% HCl was filled in a glass tube D, and most of the nicotine in the cigarette smoke was absorbed in the cotton plug. Bottle E was used for washing out the smoke.

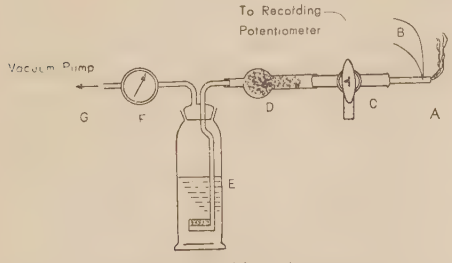

FIG. 1. Smoking Apparatus

A: Cigarette
B: Platinum and Platinum-Rhodium Thermocouple
C: Three-way Stopcock
D: Absorbent Cotton of ca. 5 g. impregnated with 5% HCl.
E: Washing Bottle Contg. H_2O
F: Flowmeter
G: Vacuum Pump

Measurement of Combustion Temperature The combustion temperature of cigarettes were measured by means of a platinum and platinum (87%)-rhodium (13%) thermocouple which was inserted into a position of 25 mm from the lighted -end of cigarette and connected with precisely responsive autorecording potentiometer*, especially deviced for the purpose of the studies of this series. After the wire was inserted at this position, the pin hole pireced by the needle in the cigarette paper was sealed with Duco cement. Twenty readings were made for each study and the reliable temperatures were calculated at a confidence level of 95%.

Selection of Thermocouples For selection of the proper size of the thermocouple, peak temperatures of the combustion zone were measured with thermocouple wires of 0.03 mm, 0.05 mm and 0.2 mm-diameters under the conditions of 20 ml/sec puff velocity, and two-second durations, twice per minute.

Determination of Nicotine in Cigarette Smoke After two cigarettes were smoked, the filling in tube D and the washed liquid of cock C in Fig. 1 were combined and steam-distilled according to the method of Griffith and Jeffrey[15], and the distillate was then subjected to spectrophotometric determination of nicotine as described by Willits[16]. Each value reported is the average of five experiments.

RESULTS AND DISCUSSION

Selection of Proper Thermocouple wire The average combustion temperature obtained with 0.05 mm-wire was measured as 812±11.1°C, and was not significantly different from 815±15.9°C obtained 0.03 mm-wire. However, the average temperature of the 0.2 mm-wire

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TABLE I. PEAK TEMPERATURES OBTAINED WITH DIFFERENT DIAMETER THERMOCOUPLES.

Diameter of wires (mm)	Peak Temperatures (°C)
0.03	815±15.9
0.05	812±11.1
0.2	656±14.6

Each value is the average obtained from twenty experiments.

TABLE II. COMBUSTION TEMPERATURES CAUSED BY DIFFERENT SMOKING PROCEDURES.

Puff Vel. (ml/sec.)	Av. Combustion Temperature Intermittent puffing (°C)	Continuous puffing (°C)
without puffing		746±31.7
5	798±11.6	794±25.3
10	769± 9.2	797±26.1
20	812±11.1	813± 8.9
40	805±10.8	827±21.1
60	802±19.0	804±13.6

Weight range of cigarette: 0.960 g ±13 mg
Moisture content: 10.93%
Room temperature: 19.4°C±1°C
Relative humidity: 59.7%±1.6%
Thermocouple: 0.05 mm diameter platinum and platinum-rhodium thermocouple wires.
Confidence level of average values of measured combustion temperature is 95%.

Each value is the average obtained from twenty experiments.

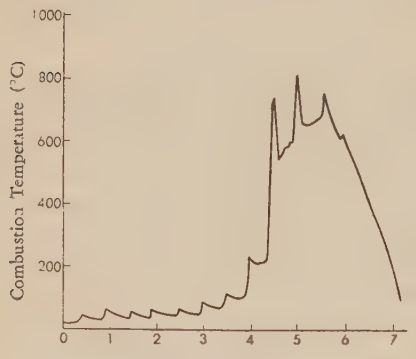

FIG. 2. Combustion Temperature-Time Curve.
(Intermittent Puffing at 20 ml/sec. Puff Velocity)

showed a remarkably low value such as 656±14.6°C. This fact may suggest that the wires of less than 0.05mm in diameter would give a more accurate temperature for the measurement of combustion-zone temperature of cigarette. These results are shown in Table I. Therefore, 0.05 mm-diameter thermocouple wires were used throughout subsequent experiments.

Effect of Smoking Procedure on Combustion Temperature As shown in Table II, the free-burning temperature was 746±31.7°C, but the combustion temperatures accompanied with puffing were within the limits of 794±25.3°C~827±21.1°C, irrespective of puff velocities and smoking types, with one exception of 769± 9.2°C at the intermittent puffing of 10 ml/sec. The result that the manner of smoking has no marked influence on combustion temperature is in accordance with the findings of Touey[7]. This may suggest that the amount of oxygen provided has no marked effect on combustion temperature; thus, the puff velocities or smoking types have no significant effect on combustion temperature. However, the combustion temberatures measured in this experiment were about 80°C lower than 873~890°C as measured by Touey[7]. The typical shape of combustions temperature-time is also given in Fig. 2. The reason why the value obtained in this study were lower than that obtained by Touey, and combustion temperature at 10 ml/sec intermittent puffing was measured lower than others, must be revealed by further studies.

Nicotine in Cigarette Smoke The amount of nicotine in cigarette smoke at different puff velocities and types of smoking are presented in Table III, and the relation of these results are shown in Fig. 3. These

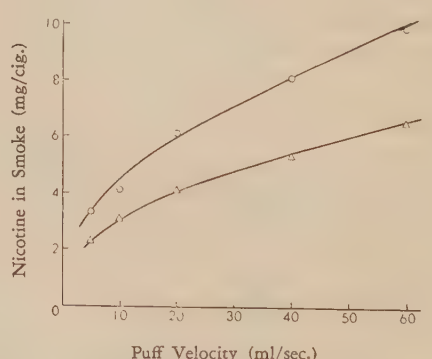

FIG. 3. Nicotine Content in Cigarette Smoke at Different Smoking Procedures.
△: Intermittent Puffing
○: Continuous Puffing

values were calculated as milligrams per one cigarette. When the cigarettes are smoked up to a definite length from the lighted end, 45 mm, the amount of nicotine transferred into cigarette smoke showed a rise in an

TABLE III. NICOTINE CONTENT IN CIGARETTE SMOKE AT DIFFERENT SMOKING.

Puff Vel.	Puff Number[a]	Nictone in smoke[b]		Nicotine in smoke vs. Nicotine in burnt portion of cigarette		Time required for 45 mm. burning on contin. puffing[c]	Time required for 1 mm burning*	The length of burnt portion for 1 second	Smoked length in an actual puff action at intermittent puffing**
		Interm.	Contin.	Interm.	Contin.				
(ml/sec.)		(mg)	(mg)	(%)	(%)	(sec.)	(sec.)	(mm)	(mm)
5	20.0	2.38	3.29	13.7	19.0	110.3	2.45	0.41	16.4
10	16.5	3.07	4.10	17.7	23.7	65.6	1.46	0.69	22.8
20	13.5	4.05	6.16	23.4	35.6	41.0	0.91	1.10	29.8
40	10.5	5.40	8.12	33.2	47.0	28.5	0.63	1.58	33.2
60	9.5	6.65	9.90	38.5	57.2	26.2	0.58	1.72	32.5

a) c) Each value is the average obtained from twenty experiments.
b) Each value is the average obtained from five experiments.

almost linear curve, from 2.38 mg (5 ml/sec) to 6.65 mg (60 ml/sec) for intermittent puffing. In the case of continuous puffing, the rise was from 3.29 mg (5 ml/sec) to 9.90 mg (60 ml/sec).

As shown in Table III (8th column)*, in case of continuous puffing, the time required for burning of 1 mm-length of cigarette during smoking-action was shortened with increment of puff velocity from 2.45 sec (5 ml/sec) to 0.58 sec (60 ml/sec). This may be a cause of the results obtained, that is, the time required for nicotine is subjected to thermal decomposition, diminishes less and less with the increase in puff velocity, therefore, the amount of pyrolyzed nicotine decreases with the increase in puff velocity. On the other hand, as the changes in puff velocity and the smoking type did not have effect on the combustion temperature of cigarettes, the increment of the amount of nicotine in cigarette smoke with the increase in puff velocity may be assumed that the time in which nicotine is subjected to thermal decomposition during actual puffing action, decreases according to increase in puff velocity.

In the case of intermittent puffing, as shown in Table III (10th column)**, the consumed cigarette length during actual puff action was not constant but varied from 16.4 mm (5 ml/sec) to 32.5 mm (60 ml/sec), according to the increase in puff velocity. Consequently, it is expected that the amount of nicotine transferred into the main stream increases with the increase in puff velocity, while the dispersed amount of nicotine into the side stream decreases.

In the case of intermittent puffing, it is therefore considered that the increase in the amount of nicotine in cigarette smoke with increase of puff velocity is caused by altering smoked-length of the cigarette during actual puff-action, besides a similar reason to be explained for continuous puffed smoking.

* These values were derived from the consumed cigarette length, 45 mm., and values shown in the 7th column of Table III.
** These values were derived from the values given in the 2nd column and 9th column, of Table III.

SUMMARY

The work described in this paper has been undertaken to clarify the effects of smoking procedures on combustion temperature of cigarettes and the nicotine content in smoke of domestic commercial cigarettes. Intermittent and continuous smoking were carried out under different puff velocities by means of an artificial smoking apparatus. The measurement of combustion-zone temperatures developed during from 5 ml/sec. to 60 ml/sec. puff velocities were made by 0.05 mm.-diameter thermocouples made of platinum and platinum (87%)-rhodium (13%) wire and by an excellent responsive autorecording potentiometer. These values were 794±25.3°C ~827±21.1°C, irrespective of smoking procedures, but with an exception of 769±9.2°C of 10 ml/sec. intermittent puffing. However, the free-burning temperature was 746±31.7°C.

When the cigarette was consumed up to 45 mm. in length from the lighted end, the nicotine content in cigarette smoke increased with puff velocity. These values rised linearly from 2.38 mg (5 ml/sec.) to 6.65 mg (60 ml/sec.) for intermittent puffing, and from 3.29 mg (5 ml/sec.) to 9.90 mg (60 m/sec.) for continuous puffing.

Acknowledgements The authors are indebted to Prof. Yataro Obata, Department of Agricultural Chemistry, Hokkaido University, for his invaluable advice and encouragement. Acknowledgements are made to Mr. Sadao Nakamura, Director of this Institute, and to Dr. Isao Ōnishi, Chief of this Department, for their encouragement, and permission to publish this work. Thanks are also due to Miss C. Tokura and Mrs. H. Hōshaku for their technical assistance.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 6, p. 532~535, 1959]

On the Influence of Moisture Content in Cigarettes on Combustion Temperature and Transferred Amount of Nicotine into Cigarettes Smoke*

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Received March 12, 1959

The effects of moisture content of cigarettes on both combustion temperature and the amount of nicotine transferred into the smoke were studied under different smoking conditions. The combustion temperatures of domestic commercial blended cigarettes were not affected by smoking procedures or amount of moisture in the cigarette. No significant differences in the amount of nicotine transferred into smoke were observed between the cigarettes with medium (10.9%) and high (15.4%) moisture contents, while the values obtained from the low moisture content (6.6%) cigarettes were always slightly higher than those obtained from medium or high moisture content cigarette.

In the author's previous study on cigarette smoke[1], it has been shown that combustion temperature of cigarettes are not affected by either puff velocities or smoking types i. e., intermittent and continuous puffing; but, in contrary to this, the amount of nicotine contained in the smoke, shows a rise approaching a linear-curve in accordance with the increase in puff velocity.

It has long been recognized that the smoke of cigarettes having a low moisture content is more irritant to the throat than that of cigarettes having a high moisture content[2,3], and that the amount of nicotine contained in the smoke of dry cigarettes is comparatively richer than that of moist ones[4]. However, no literature dealing with the effect of the moisture content of cigarettes on combustion temperature and on the amount of nicotine transferred into smoke under different smoking conditions, has yet been found. The present study was performed in order to clarify the problem of thermal decomposition of nicotine in tobacco and the transfer of alkaloids into the smoke.

EXPERIMENTAL

Preparation of Sample and Smoking Conditions Sample cigarettes of 70 mm Standard length, blended

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TABLE I. COMBUSTION TEMPERATURE OF CIGARETTES OF DIFFERFNT MOISTURE CONTENTS

Puff Vel.	Intermittent Puffing Moisture Content (%)			Continuous Puffing Moisture Content (%)		
	6.61	10.93	15.40	6.61	10.93	15.40
(ml/sec.)	(°C)	(°C)	(°C)	(°C)	(°C)	(°C)
10	811±13.3	769± 9.2	814±18.8	804±17.3	797±26.1	807±27.2
20	799± 9.8	812±11.1	815±15.8	802± 8.9	813± 8.9	794±18.2
40	805±17.9	805±10.8	814±25.3	815±13.2	827±21.1	800±17.6

Weight Range of cigarettes: 0.960 g±13 mg at 10.93% moisture content
Room Temperature: 19.4±1°C
Relative Humidity: 59.7±1.6%
Thermocouple: a 0.05 mm -diameter, platinum and platinum-rhodium thermocouple.
Confidence level of average value of measured combustion temperature: 95%
Each value is the average obtained from twenty experiments.

with domestic flue cured, Indian flue cured, domestic naturally cured and Indian naturally cured tobaccos, were used throughout this investigation. The cigarettes were conditioned to a medium moisture content (10.9%) with a relative humidity of 58% at 20°C, then selected in a weight range of 960±13 mg. Previous to use, the cigarettes were stored for a week in an atmosphere of relative humidity 43%, 58% and 70% at 20°C, respectively. Each of the three kinds of equilibrated cigarettes had low, medium and high moisture contents, i.e., 6.61%, 10.93% and 15.40%, respectively. They were smoked up to 45 mm-length from the lighted end, in a room maintained at a relative humidity of 59.7±1.6%, and at 19.4±1.0°C. The smoking conditions were as follows: Puff interval two-seconds' duration in a half minute, except continuous puffing. Puff velocities—10 ml/sec., 20 ml/sec. and 40 ml/sec.

Apparatus and Procedures The apparatus for smoking, and an instrument employed for measurement of the combustion temperature of cigarettes were the same as those described in the previous paper[1]. The combustion temperatures were measured by means of a plantinum and plantinum-rhodium, 0.05 mm-diameter thermocouple.

The amount of nicotine contained in the smoke were determined photometrically according to the technique previously described[1].

RESULTS AND DISCUSSION

The combustion temperatures of cigarettes having different moisture contents were measured within the limits of 794±25.3°C and 827±21.1°C under various smoking conditions, with only one exception of 769±9.2°C which was obtained from medium moisture content cigarettes at 10 ml/sec. continuous puffing, as shown in Table I. On the basis of limited information obtained from this study, the results illustrate that the combustion temperature of cigarettes is not markedly affected by the moisture content or smoking procedures, i.e., puff velocity and type of smoking.

The effect of moisture content in cigarettes on the amount of nicotine transferred into smoke under different smoking procedures is illustlated in Table II and Fig. 1. The amount of nicotine in the smoke rises linearly with the increase in puff velocity, and varies from 3.07 mg (10 ml/sec puff velocity) per cigarette to 5.4 mg (40 ml/sec), for medium moisture cigarettes.

TABLE II. AMOUNT OF NICOTINE IN CIGARETTE SMOKE OBTAINED UNDER DIFFERENT SMOKING CONDITIONS

Puff Vel.	Intermittent Puffing Moisture Content (%)			Continuous Puffing Moisture Content (%)		
	6.61	10.93	15.40	6.61	10.93	15.40
(ml/sec.)	(mg)	(mg)	(mg)	(mg)	(mg)	(mg)
10	3.37	3.07	2.98	5.49	4.10	4.05
20	4.69	4.05	4.10	7.36	6.16	5.58
40	6.42	5.40	5.41	9.14	8.12	7.41

Each value is the average obtained from five experiments.

Although the differences of the moisture contents between both of high-medium and medium-low cigarettes were almost equal, no significant differences on the amount of nicotine transferred were observed between medium and high moisture cigarette, but the value obtained from low moisture cigarettes were always slightly higher than those obtained from medium and high moisture cigarettes. As the nicotine has long been recognized as being subjectively extremely irritating to the throat, the result agree well with the fact that dry cigarette more irritate the throat than moistend cigarette. This evidence may suggest that the lower content of

moisture as compared with the medium moisture content, has a definite effect on the increase in the amount of nicotine transferred into the smoke, while the moisture content of cigarettes higher than the medium has only little effect.

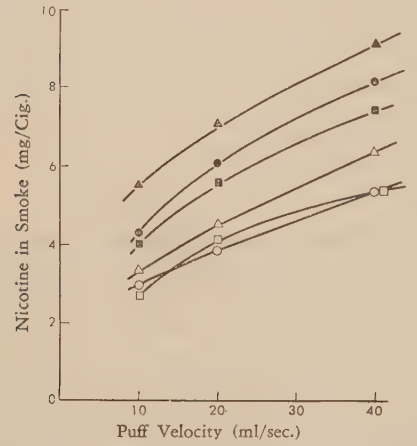

FIG. 1. Nicotine Content in Cigarette Smoke Produced by Burning Cigarette Having Different Moisture Contents.

△: Cigarette of 6.61 % Moisture Content
○: 〃 10.93 % 〃
□: 〃 15.40 % 〃

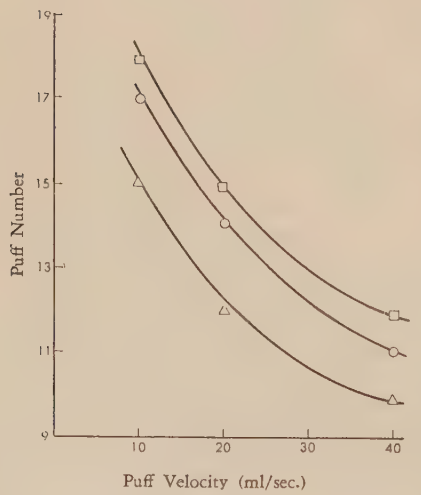

FIG. 2. Puff Number of Cigarettes Having Different Moisture Content.

△: Cigarette of 6.61 % Moisture Content
○: 〃 10.93 % 〃
□: 〃 15.40 % 〃

Similar trends as those described above were also observed in the case of continuous puffing (See Table II and Fig. 1).

TABLE III. PUFF NUMBER AND TIME REQUIRED FOR BURNING OF CIGARETTES WITH DIFFERENT MOISTURE CONTENTS.

Puff Vel.	Puff Number			Time Required for 45 mm Burning		
	Moisture Content (%)			Moisture Content (%)		
	6.61	10.93	15.40	6.61	10.93	15.40
(ml/sec.)				(sec.)	(sec.)	(sec.)
10	15	17	18	56.5	65.6	70.1
20	12	14	15	37.5	41.0	43.0
40	10	11	12	24.7	28.5	32.3

Each value is the average obtained from twenty experiments.

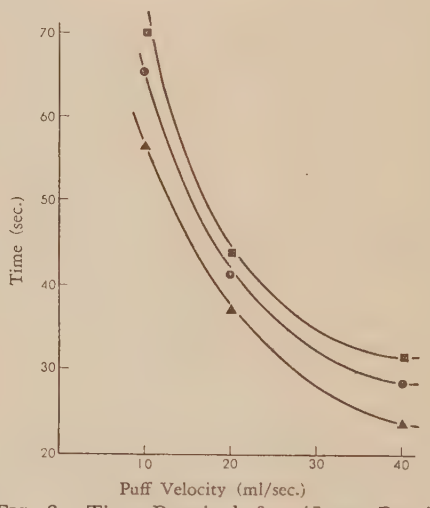

FIG. 3. Time Required for 45 mm Burning of Cigarette Having Different Moisture Content.

△: Cigarette of 6.61 % Moisture Content
○: 〃 10.93 % 〃
□: 〃 15.40 % 〃

The puff number at intermittent puffing, and time required for 45 mm-burning at continuous puffing taken on three cigarettes moistened in various degrees, are represented as a function of puff velocity in Table III, and Fig. 2,3. For instance, when the cigarettes were smoked under 10 ml/sec puff velocity, the differences of puff number among these three cigarettes were 2, between low and medium moisture cigarettes, but 1 between medium and high moisture cigarettes. The differences of time required for 45 mm-burning among

these three various moisture cigarettes were 9.1 seconds between low and high moisture cigarettes, but only a period of 4.5 seconds was required between medium and high moisture cigarettes. A similar trend was observed in case of every puff velocity.

Consequently, it is evident that moisture exceeding a medium content does not give a significant effect on the puff number and burning time, but moisture less than the medium level has a significant effect on the puff number and time required for 45 mm-burning.

On the contrary as the combustion temperatures were almost constant regardless of the moisture content in cigarettes, the variation of the amounts of transferred nicotine may be mainly attributed to the variation of puff number and of burning time which are caused by variation of moisture content in cigarettes.

SUMMARY

1) The combustion temperatures of domestic commercial blended cigarettes are not affected by the moisture content in cigarettes or the smoking procedure, and, most of these values are within the limits of 794±25.5°C and 827±21.1°C.

2) No significant differences in the amount of nicotine transferred into smoke were observed between 10.9% and 15.4% moistured cigarettes, while values obtained from low moistured, 6.6%, cigarettes, were always slightly higher than those obtained from both 10.9% and 15.4% moistured cigarettes.

Acknowledgements The authors wish to express their appreciation to Professor Yataro Obata, Department of Agricultural Chemistry, Hokkaido University, during the course of this investigation. The authors wish to thank Mr. Sadao Nakamura, Director of this Institute, and Dr. Isao Ōnishi, Chief of this Department, for their encouragement and permission to publish this work. Thanks are also due to Mrs. H. Hōshaku and Miss M. Watanabe for their technical assistance.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 6, p. 536~547, 1959]

Chemical Studies on the Antibiotic Esperin
Part III. On the Structure of Esperin

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Received March 14, 1959

Esperin is an acidic antibiotic with a molecular formula of $C_{39}H_{67}N_5O_{11}$ and, on hydrolysis with acid, it afforded L-aspartic acid, L-glutamic acid, L-valine, L-leucine, D-leucine and 2-tridecenoic acid. By treatment with alkali, esperin was transformed to esperinic acid, $C_{39}H_{69}N_5O_{12}$, which was shown to be β-hydroxytridecanoyl-glutamyl-aspartyl-valyl-leucyl-leucine. From chemical and physical studies, esperin was proved to be the lactone of esperinic acid, represented by the formula III.

Esperin is an antibiotic which is produced by a variety of *Bacillus mesentericus*. In the previous papers[1,2], it was shown that esperin was a polypeptide which gave by hydrolysis, DL-leucine, L-aspartic acid, L-valine and 2-tridecenoic acid, and was easily transformed in good yield to a crystalline compound, sodium salt of esperinic acid[3]. Esperinic acid was found to be less active against *Mycobacterium tuberculosis*, but less toxic than esperin.

The present paper reports experiments designed to determine the structure of esperin.

Esperin has hitherto been difficult to crystallize. But when examined by paper chromatography and by countercurrent distribution method, it appeared to consist of one active component, and its further purification was carried out by repeated precipitation from acetone-petroleum ether, and finally was crystallized from isopropanol-petroleum ether.

Crystalline esperin had $[\alpha]_D^{15}-24°$ (c, 0.66% in methanol), and the melting point of 238° and was found to contain carbon, hydrogen, nitrogen and oxygen. Its molecular weight in camphor (micro-Rast) was 730~800.

The most probable empirical formula based on the analysis was $C_{39}H_{67}N_5O_{11}$, which was also supported by the degradation studies as described in this paper.

Esperin is soluble in methanol, ethanol, chloroform; moderately soluble in acetone; but insoluble in water, ether and petroleum ether, and is an acidic compound which is soluble in aqueous solution of sodium hydroxide and sodium bicarbonate.

Esperinic acid crystallized in long needles from acetone-petroleum ether mixture, had $[\alpha]_D^{15}+12.5°$ (c, 1.6% in methanol), the melting point of 195°, and its sodium salt melted at 269°. It is of interest to note that esperin was levorotatory while esperinic acid was dextrorotatory in methanol solution. The molecular weight of esperinic acid was estimated by the Rast and by the Signer methods, each corresponding to 1000~1100 and 830~920 respectively. Analysis of esperinic acid indicated a probable molecular formula of $C_{39}H_{69}N_5O_{12}$.

Esperin and esperinic acid showed only end-absorption in the ultraviolet spectra and took up no hydrogen in the presence of platinum catalyst. The absence of primary amino group was proved by the Van Slyke nitrous acid determination, potentiometric titration and a negative ninhydrin reaction.

The titration of esperin and esperinic acid in

1) H. Ogawa and T. Ito, *J. Agr. Chem. Soc. Japan*, **24**, 191 (1951).
2) H. Ogawa and T. Ito, *J. Agr. Chem. Soc. Japan*, **26**, 432 (1952).
3) In the previous papers, we had named this compound as Esperin X.

absolute methanol with 0.1N tetrabutylammonium hydroxide in methanol (indicator; thymolblue)[4] indicated that the equivalent weight of esperin was 450 and that of esperinic acid was 330. From these results, it was thought that esperin was dibasic and esperinic acid tribasic acid.

The above data were summarized as below,

Esperin	NaOH or KOH → in ethanol	Esperinic acid
$C_{39}H_{67}N_5O_{11}$		$C_{39}H_{69}N_5O_{12}$
(M.W. 782)		(M.W. 800)
m.p. 238°		m.p. 195°
$[\alpha]_D^{15}$ −24° (in MeOH)		$[\alpha]_D^{15}$ +12.5° (in MeOH)
Dibasic		Tribasic

These results suggested that the conversion of esperin to esperinic acid with alkali might be the opening of lactone ring of the former. To confirm this supposition their infrared spectra were examined. The infrared absorption spectra of esperin, esperinic acid and their sodium salts are shown in Fig. 1~4. The amide I and amide II absorptions of esperin appeared at 1639 cm^{-1} and 1538 cm^{-1}, and those of esperinic acid at 1618 cm^{-1} and 1543 cm^{-1}. These bands should be assigned to the absorptions of -CONH- groups. The absorption of esperinic acid at 1695 cm^{-1} indicated undoubtedly −C=O vibration of carboxyl groups and in the spectrum of its sodium salt, this carboxyl absorption vanished and was replaced by two bands at 1575 cm^{-1} and 1408 cm^{-1}, which should correspond to the antisymmetrical and symmetrical vibrations of the $-COO^-$ structure[5]. This fact indicated that esperinic acid had carboxyl groups but no ester group.

Esperin gave absorption at 1735 and 1720 cm^{-1}, which were due to –C=O vibration of ester and carboxyl. And the sodium salt of esperin[6] showed absorptions at 1575, 1400 and 1730 cm^{-1}. The bands at 1575 and 1400 cm^{-1} corresponded to vibrations of $-COO^-$ structure and the band at 1730 cm^{-1} indicated –C=O vibration of ester. These data indicated that esperin had both carboxyl groups and an ester group.

The presence of hydroxyl group in esperinic acid was assured also by the following infrared absorption spectrum. Esperinic acid was acetylated with acetic anhydride and fused sodium acetate. The resulting acetate, though could not be obtained in crystalline form, the infrared spectrum of its sodium salt showed the presence of O-acetyl group.

Esperin gave positive ferric hydroxamate test[7] of ester group, whereas esperinic acid was negative.

4) R. H. Cundiff and P. C. Markunas, *Anal. Chem.*, **28**, 792 (1956).

5) L. J. Bellamy, The Infrared Spectra of Complex Molecules, p. 149 (1954) (Methuen, London)

6) This salt was prepared by treatment with sodium bicarbonate and was determined to have no free carboxyl group by potentiometric titration.

7) N. D. Cheronis, Technique of Organic Chemistly, VI, 460 (1954), Interscience Pub.

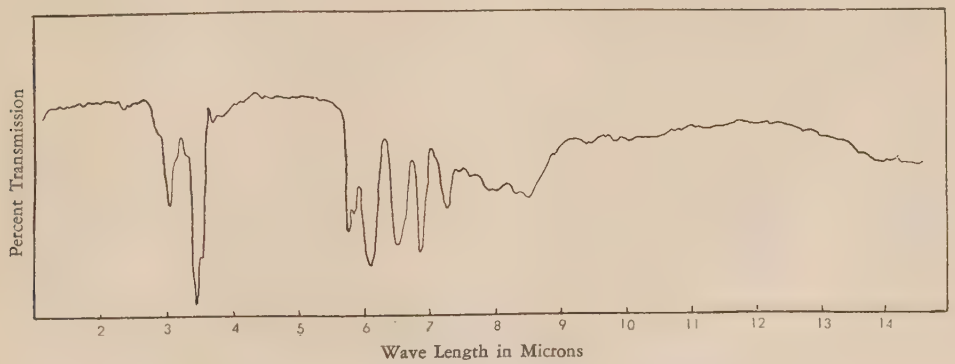


FIG. 1. Infrared Spectrum of Esperin in Nujol

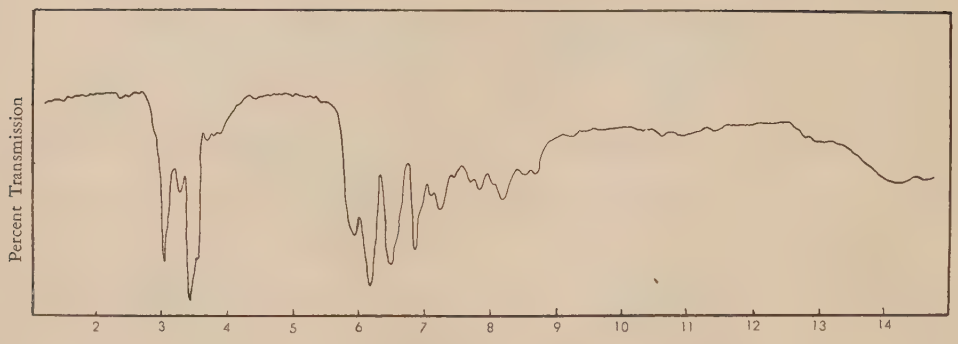


FIG. 2. Infrared Spectrum of Esperinic Acid in Nujol

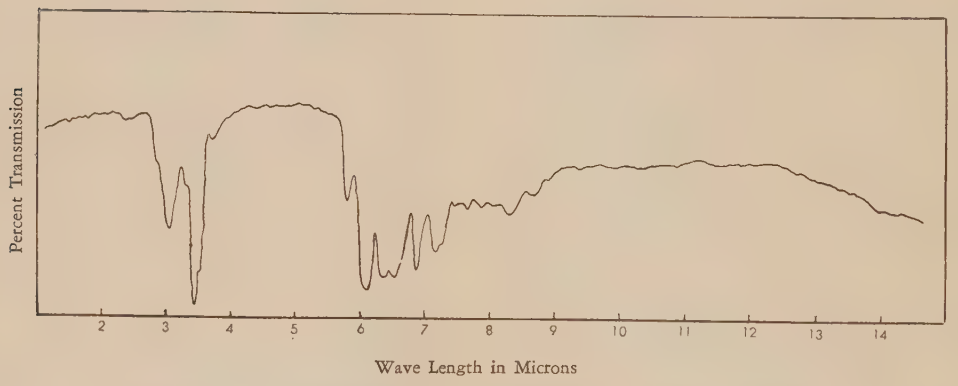


FIG. 3. Infrared Spectrum of Sodium Salt of Esperin in Nujol

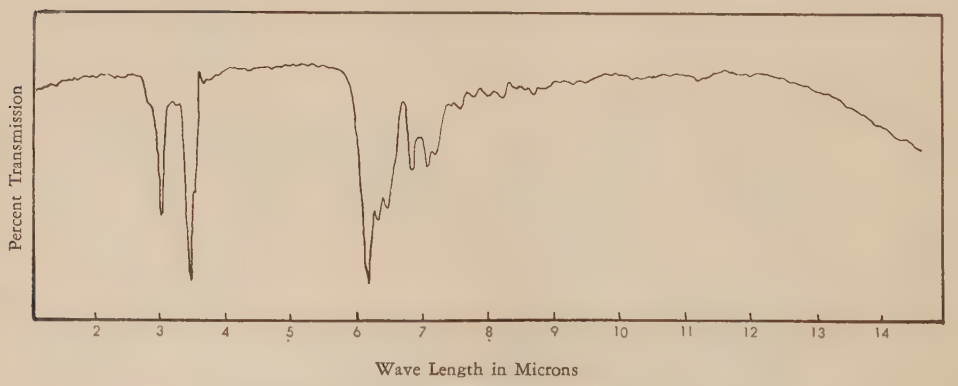


FIG. 4. Infrared Specrum of Sodium Salt of Esperinic Acid in Nujol

Amino Acid Composition of Esperin.

In the previous report[1], it was shown that, on descending paper chromatograms with a mixture of *n*-butanol and water as solvent, the acid hydrolysates of esperin and esperinic acid gave three ninhydrin-positive spots. Their R_F values coincided with those of aspartic acid, valine and leucine, and their identity were further determined by the isolation of these amino acids.

The amino acid composition of esperin was later reexamined by paper chromatography with other solvent systems. The ascending paper chormatograms, with a solvent mixture consisting of *n*-butanol, acetic acid and water (4 : 1 : 2.5 by vol.), showed four spots. This chromatogram indicated the presence of leucine, valine, aspartic acid and also another amino acid. The fourth amino acid had the same R_F value as that given by glutamic acid. It was thought that the authors had failed to notice the presence of glutamic acid in the previous paper[1], for glutamic acid had almost identical R_F value with aspartic acid on paper chromatograms with *n*-butanol-water solvent system.

This amino acid was isolated as crystalline hydrochloride by boiling esperin with concd. HCl, concentrating and saturating with hydrogen chloride. This amino acid hydrochloride was identical with an authentic sample of L-glutamic acid hydrochloride on comparison of infrared spectrum, optical rotation, melting point and R_F values on paper chromatograms.

The R_F values of amino acids in esperin obtained by one-dimensional paper chromatography with four solvent systems are shown in Table I. Two-dimensional paper chromatogram (solvent: *n*-butanol, acetic acid water and 80% phenol) showed also four spots.

The quantitative relationship of the amino acid residues in the molecule of esperin was determined with paper chromatograms, by using commercial photoelectric transmission densitometer. The molar ratio of amino acid residues was leucine 2, valine 1, glutamic acid 1 and aspartic acid 1.

By observing optical rotations, aspartic acid, glutamic acid and valine were designated to be L-isomers, whereas leucine was racemic. It was believed that both L and D-isomers of leucine should be present in the molecule of esperin, because an optically active leucine was hardly raceminized under the above condition of hydrolysis.

The amino acid composition of esperinic acid was also found identical with that of esperin.

The amino acid composition of esperinic acid was further confirmed by measuring the optical rotation of its hydrolysate. The hydrolysate of esperinic acid, after removing fatty acid, had identical optical rotation with that of the mixture of authentic L-aspartic acid, L-glutamic acid and L-valine, which were equimolar to esperinic acid.

By the hydrolysis of esperin and esperinic acid, no ammonia was liberated.

The above experiments indicated that the

TABLE I. THE R_F VALUES OF AMINO ACIDS IN ESPERIN BY ONF-DIMENSIONAL PAPER CHROMATOGRAPHY (Ascending method)

Amino Acid	Solvent* 1	2	3	4
Aspartic acid	0.27 (0.28)**	0.11 (0.09)	0.04 (0.04)	0.02 (0.02)
Glutamic acid	0.33 (0.34)	0.22 (0.17)	0.04 (0.04)	0.04 (0.04)
Valine	0.56 (0.55)	0.75 (0.79)	0.28 (0.29)	0.23 (0.21)
Leucine	0.70 (0.71)	0.82 (0.86)	0.42 (0.45)	0.37 (0.37)

* Solvent: 1. *n*-butanol, acetic acid, H_2O (4 : 1 : 2.5 V)
2. 80% phenol
3. 2-butanol, 3% NH_3 (3 : 1 V)
4. *n*-butanol, H_2O (85 : 15 V)
Coloring Reagent: Ninhydrin

** The R_F values in parentheses are those of the standard amino acids.

molecule of esperin (and esperinic acid) was composed of five amino acid residues and one fatty acid residue (C_{13}).

These results are summarized in Table II.

Table II. The Composition of Esperin (and Esperinic acid)

		Number of residue
L-Aspartic acid	$C_4H_7NO_4$	1
L-Glutamic acid	$C_5H_9NO_4$	1
L-Valine	$C_5H_{11}NO_2$	1
L-Leucine	$C_6H_{13}NO_2$	1
D-Leucine	$C_6H_{13}NO_2$	1
Fatty acid (C_{13}) (β-Hydroxytridecanoic acid)		1
Esperin	$C_{39}H_{67}N_5O_{11}$	
Esperinic acid	$C_{39}H_{69}N_5O_{12}$	

An open chain polypeptide composed of these amino acids and a fatty acid would have three free carboxyl groups and no amino group. Actually esperin and esperinic acid have no amino group, showing negative reaction with 2,4-dinitrofluorobenzene.

Carboxyl-terminal Residue.

The analysis of C-terminal amino acid in esperin and esperinic acid was performed by the next three methods: the hydrazine method of Akabori et al.[8], the reaction with acetic anhydride and pyridine (Turner's method), and enzymatic degradation with carboxypeptidase.

The hydrazine method: Akabori, Ohno and coworkers[8] proposed hydrazinolysis as a method for the identification of C-terminal amino acids. Several modifications of this method were reported[9,10], and in the present work the method used by K. Kusama[9] was applied.

The results thus obtained showed that both esperin and esperinic acid has leucine as the C-terminal amino acid. Besides, the results also indicated the presence of groups $-NH-\underset{\displaystyle (CH_2)_{1-2}\,COOH}{\underset{|}{CH}}-CO-$ and (or) $-NH-\underset{\displaystyle (CH_2)_{1-2}\,CO-}{\underset{|}{CH}}-COOH$ in the molecule.

Turner's method: R.A. Turner and G. Schmerzler[11] reported a method for the identification of C-terminal amino acid, by using Dakin-West reaction of amino acid as follows.

$$-NHCHR^1CONHCHRCOOH \xrightarrow[C_5H_5N]{(CH_3CO)_2O} CO_2 + -NHCHR^1CONHCHRCOCH_3$$

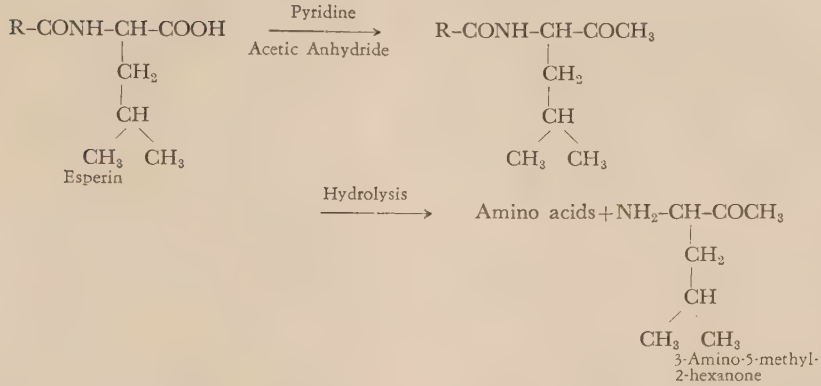
$$\xrightarrow{H_2O} + NH_2CHR^1COOH + NH_2CHRCOCH_3$$

Esperin, acetic acid anhydride and pyridine were heated in a sealed tube at 150° for 2.5 hours. After the solvent was removed in vacuo, the residue was hydrolyzed and was chromatographed on paper with *n*-butanol, acetic acid and water mixture (4 : 1 : 2.5 V).

The chromatogram, sprayed with ninhydrin, showed five spots, of which R_F values were 0.27, 0.33, 0.56, 0.70 and 0.75. The spots of R_F 0.27, 0.33, 0.56 and 0.70 were undoubtedly due to L-aspartic acid, L-glutamic acid, L-valine and leucine. The R_F value of 0.75 was coincided with that of 3-amino-5-methyl-2-hexanone, which was derived from leucine by Dakin-West reaction. So the above reaction of esperin could be described as the next equation.

And from the mechanism of Dakin-West reaction[12] and from the fact that L-aspartic acid and L-glutamic acid in esperin were not destroyed by this reaction, it was clear that α-carboxyl groups of L-glutamic acid and L-aspartic acid were not free but were bound to other amino acids with peptide bonds.

8) S. Akabori, K. Ohno and K. Narita, *Bull. Chem. Soc. Japan*, **25**, 214 (1952).
K. Ohno, *J. Biochem.* (Japan), **40**, 621 (1953).
K. Ohno, *J. Biochem.* (Japan), **41**, 345 (1954).
9) K. Kusama, *J. Biochem.* (Japan), **44**, 375 (1957).
10) Gerhard Braunitzer, *Chem. Ber.*, **88**, 2025 (1955).
11) R.A. Turner and G. Schmerzler, *J. Am. Chem. Soc.* **76**, 949 (1954).
12) G.H. Cleland and C. Niemann, *J. Am. Chem. Soc.*, **71**, 841 (1949).



(R. A. Turner and G. Schmerzler[11]) reported that aspartic acid in phthaloyl-β-alanyl-DL-asparagine was destroyed by this reaction.) This reaction when tested with esperinic acid showed the same results as with esperin.

Reaction with carboxypeptidase: Esperin and esperinic acid were unaffected with carboxypeptidase. It is well established[13,14,15,16] that the peptide having the C-terminal amino acid of D-configuration is not attacked by carboxypeptidase. So this fact may suggest that the C-terminal leucine in esperin (and esperinic acid) is D-isomer.

In order to ascertain this view, esperin was reacted with acetic anhydride and pyridine, and then the reaction product was hydrolyzed. From its hydrolyzate, leucine was isolated by a procedure similar to that already described[1] and its specific rotation was measured. Since the isolated leucine was L isomer, C-terminal leucine which was degenerated by the Turner's reaction was D isomer.

Amino Acid linked to Fatty Acid.

Esperinic acid was hydrolyzed partially by heating in 12 N hydrochloric acid at 100° for 3 hours. The ether-soluble substance was separated by extraction with ether and was hydrolyzed completely by boiling in 12 N hydrochloric acid for 5 hours. The paper chromatogram of this hydrolyzate gave one definite spot of L-glutamic acid. And so the amino acid linked to fatty acid was established to be L-glutamic acid.

Partial Hyrolysis of Esperinic Acid.

The partial hydrolysis of esperinic acid was investigated using various concentrations of hydrochloric acid. The best condition of partial hydrolysis appeared to be standing the solution of esperinic acid in the mixture of 12N hydrochloric acid and glacial acetic acid (1 : 1 V.) at 35° for 1~2 weeks. Peptides formed were separated by ionic exchange resin column chromatography (Amberlite IR~4B) and paper chromatography. The amino acid residues in the peptides were determined by complete hydrolysis and the N-terminal residues by reaction with dinitrofluorobenzene.

A neutral peptide, which showed R_F value 0.85 on paper chromatogram (solvent: *n*-butanol, acetic acid, H_2O 4 : 1 ; 2.5), gave only leucine by complete hydrolysis. This peptide gave R_F value 0.87 on paper developed with phenol-water, which was almost coincided with that of L-leucyl-D-leucine, which had been described in the reports[17,18].

A peptide of R_F 0.93 (solvent: *n*-butanol,

13) Shou-Cheng J. Fu, Sanford M. Birnbaum and Jesse P. Greenstein, *J. Am. Chem. Soc.*, **76**, 6054 (1954),
14) H. T. Hanson and E. L. Smith, *J. Biol. Chem.*, **179**, 815 (1949).
15) M. A. Stahmann, J. S. Fruton and M. Bergmann, *ibid.*, **164**, 753 (1946).
16) Sam Yanari and Milton A. Mitz, *J. Am. Chem. Soc.*, **79**, 1150 (1957).
17) J. I. Hariss, P. D. Cole and N. G. Pon, *Biochm. J.*, **62**, 154 (1956).
18) C. A. Knight, *J. Biol. Chem.*, **190**, 753 (1951).

acetic acid, H_2O 4 : 1 : 2.5 V.) contained leucine, L-valine and L-aspartic acid and its N-terminal residue was shown to be L-aspartic acid by DNP method. And an acidic peptide of R_F 0.18 was composed of L-aspartic acid and L-glutamic acid.

These experiments were summarized and the next sequence was deduced.

Fatty acid→L. glu.→L. asp.→L. val.→L. leu.→D. leu
Abbreviation :→–CONH–; glu. glutamic acid; asp. aspartic acid; val. valine; leu. leucine.

The Structure of Esperinic Acid.

It was observed that esperinic acid had no double bond, but the fatty acid obtained after hydrolysis of esperinic acid with concd. hydrochloric acid, was α, β-unsaturated tridecenoic acid. So this double bond in the fatty acid should be subsequently derived by dehydration during hydrolysis, for esperinic acid was shown to have a hydroxyl group. It is also well known that β-hydroxy acid can easily undergo the dehydration to α, β-unsaturated acid.

All these results could therefore be presented in the following structure I.

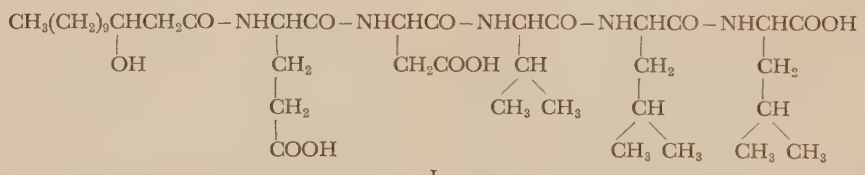
The Structure of Esperin.

As esperin had lactone structure and had leucine as C-terminal residue, it must be one of the following two structures, II and III.

Potentiometric titration in 90% methanol, indicated that the apparent dissociation constants of carboxyl grups in esperin were 5.8 and 7.1, and those in esperinic acid were 5.6, 6.4 and 7.1. It was supposed that the carboxyl groups of pKá 5.6~5.8 was α –COOH group of terminal leucine and pKá 7.1 was indicative of γ –COOH group of glutamic acid. Therefore the following experiments were done to determine the amino acid which formed lactone link in esperin molecule.

Treatment of esperin with hydrazine hydrate yielded esperin monohydrazide. The hydrazide was destroyed by the Curtius reaction according to the method of J. Kovács and coworkers[19], and then, the reaction product was hydrolyzed

19) J. Kovács, V. Bruckner and K. Kovács, *J. Chem. Soc.*, **1952**, 4255; **1953**, 145.



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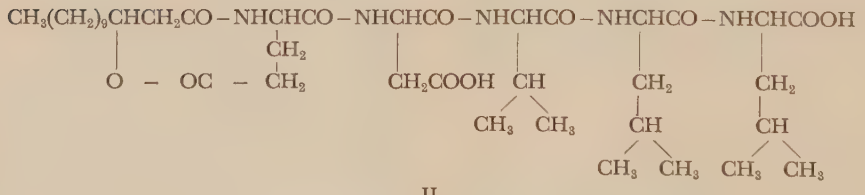
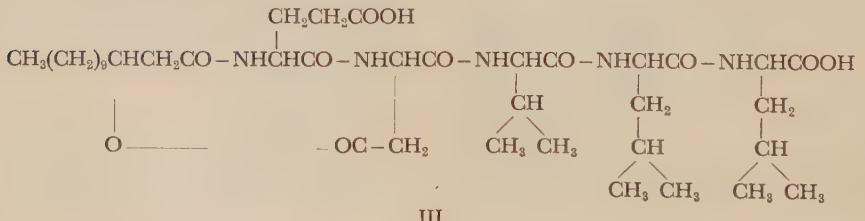
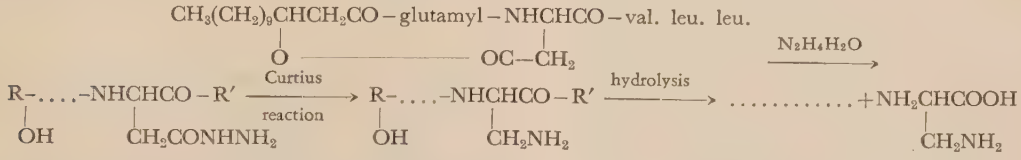


II



III

completely. The paper chromatogram of this hydrolyzate indicated that the above reaction destroyed aspartic acid and yielded an amino acid of which R_F value was coincided with that of α, β-diaminopropionic acid. The course of the degradtion is visualized as follows:



It is evident that the lactone link involves the carboxyl group of aspartic acid and the hydroxyl group of oxyfatty acid.

Partial hydrolysis of esperin described in the experimental part also supported this structure of lactone.

From the above data, the structure III was assigned to esperin.

The structure of esperin appears to resemble an antibiotic, viscosin[20], but is different from viscosin which contains no lactone structure. Actinomycin[21] and etamycin[22] are polypeptides with lactone, but they contain no fatty acid.

EXPERIMENTAL

Crystallization of Esperin—Five hundred mg of amorphous esperin were dissolved in isopropanol and the solution was concentrated in vacuo to a small volume. Petroleum ether was added carefully and the solution was kept in a refrigerator. The crystals separated slowly. (From other solvents, for example, acetone-ether mixture, ethanol-petroleum ether mixture, etc., esperin gave amorphous precipitates). The yield was 340 mg, m.p. 238°. $[\alpha]_D^{15}$ −24° (c, 0.66% in methanol).

Anal. Calcd. for $C_{39}H_{67}N_5O_{11}$: C, 59.90; H, 8.63; N, 8.95. Found: C, 60.37; H, 8.90; N, 8.86.

Molecular weight was determined by micro Rast (camphor) method.

Calcd. 782. Found. 800, 730

Esperinic Acid—Esperin (2 g) was dissolved in 10 ml of ethanol and to this were added 2 ml of 10% aqueous sodium hydroxide. The solution was allowed to stand at room temperature overnight. The crystalline sodium salt of esperinic acid was obtained by filtration. The yield was 1.7 g. Recrystallization from 70% ethanol gave needle crystals, which melted at 269°. The free acid of esperinic acid was obtained by acidifying its aqueous solution with hydrochloric acid. The resulting precipitate was removed by filtration, dried and, recrystallized from acetone-petroleum ether mixture. Long needle crystals, m.p. 195°. $[\alpha]_D^{15}$ +12.5° (c, 1.6% in methanol)

Anal. Calcd. for $C_{39}H_{69}N_5O_{12}$: C, 58.55; H, 8.69; N, 8.75. Found: C, 57.91; H, 8.34; N, 8.99.

Molecular weight was determined by micro Rast (camphor) and Signer method. The determination by Signer method was done in the apparatus of Clark[23], using ethanol as solvent.

Calcd. 800. Found: Rast method 1000, 1100
Signer method 830, 920

Esperin could not be converted to esperinic acid by treatment with sodium bicarbonate.

Titration of Esperin and Esperinic Acid—The titration of esperin and esperinic acid in 90% methanol with 0.1 N aqueous sodium hydroxide solution (indicator, phenolphthalein) indicated that the equivalent weight of esperin was 550 and that of esperinic acid was 370. Since free esperinic acid and esperin were insoluble in water and their acidities were rather weak, the titration in nonaqueous solvent was tested. Esperin and esperinic acid were titrated in absolute methanol with 0.1 N tetrabutylammonium hydroxide in methanol[4], which was prepared from tetrabutylammoniumiodide by the reaction with silver oxide and was standardized against benzoic acid. The indicator was thymolblue in isopropanol (0.3%)[4].

Equivalent weight of esperin ; Found 450
Equivalent weight of esperinic acid; 330

Total Hydrolysis of Esperin—Esperin was hydrolyzed

20) K. Toki and T. Ohno, *J. Agr. Chem. Soc. Japan*, **29**, 370 (1955).
21) H. Brockmann et al., *Angew. Chem.*, **66**, 70 (1956).
22) J. C. Sheehan, H. G. Zachau and W. B. Lawson, *J. Am. Chem. Soc.*, **80**, 3349 (1958).
23) E. P. Clark, *Ind. Eng. Chem. Anal. Ed.*, **13**, 820 (1941).

by boiling for 8 hours with 12 N hydrochloric acid with reflux condenser. 2-Tridecenoic acid separated oily during hydrolysis was extracted with ether. The yield of this acid was 20~30% of esperin. The water layer was concentrated in vacuo to dryness, and hydrochloric acid was removed by repeated evaporation with addition of water under reduced pressure. The resulting residue was dissolved in water (5 mg/ml) and chromatographed on paper. The standard amino acid solutions (1 mg/ml) and the hydrolyzate solution were applied with micropipets to Tōyō No. 50 paper strips by one dimensional ascending method. After the paper strips were dried, the spots were developed by dipping in ninhydrin solution. The results are shown in Table I.

Paper chromatograms of hydrolyzates of esperinic acid showed the identical amino acid composition with esperin.

Amino Acid Content of Esperin—In order to determine the ratio of amino acids in esperin a quantitative paper chromatographic method was used. One dimensional paper chromatograms of the esperin total hydrolyzate were run with *n*-butanol, acetic acid and water mixture (4 : 1 : 2.5 V.). The spots were developed by dipping in ninhydrin solution according to J.F. Roland et al.[24]. After drying, the strips were placed in the dark for 24 hours and then dipped in melted paraffin to make background transparent. The color intensities of spots were measured by commercial photoelectric transmission densitometer (manufactured by Natume Seisakusho) and their R_F-density curve areas were determined by planimeter. Quantities of amino acids were calculated from standard curves prepared from the paper chromatographic determination of the known quantities of the pure amino acids under same conditions.

This experiment gave a molar ratio of 1 : 1 : 1 : 2 for aspartic acid : glutamic acid : valine and : leucine.

L-Glutamic Acid from Esperin—Esperin (1.5 g) was suspended in 20 ml of conc. hydrochloric acid and refluxed for 4 hours at 100°. After removing tridecenoic acid by ether extraction, the solution was concentrated in vacuo to 4 ml, and saturated with hydrogen chloride gas at 0°. After standing at 0° overnight, the crystals were recovered by filtration. Yield 168 mg. This crude crystals were dissolved in 0.4 ml of water and saturated with hydrogen chloride, plate crystals being formed. Yield 86 mg, m.p. 203° (with dec.), Authentic L-glutamic acid hydrochloride m.p. 205° (with dec.), mixed m.p. 203~204° (with dec).

$[\alpha]_D^{30}$ +24° (c, 1% in H_2O), L-glutamic acid hydrochloride $[\alpha]_D^{22}$ +24.4° (in H_2O)

The infrared spectra of this substance and authentic L-glutamic acid hydrochloride were identical.

This amino acid had characteristic taste of L-glutamic acid.

Anal. Calcd. for $C_5H_9NO_4$: HCl ; N, 7.63. Found. N, 7.61.

L-Aspartic Acid, L-Valine and DL-Leucine from Esperin—The isolation of these amino acids had been reported in the previous paper[1].

Rotatory Power of Hydrolyzate of Esperinic Acid—Two hundred mg of esperinic acid (0.25 millimole) was hydrolyzed and, after removing fatty acid, was concentrated to dryness under reduced pressure. The residue was dissolved in 10 ml of 1 N hydrochloric acid, and the rotation was determined in 1 dm tube. The observed rotation $\alpha = +0.24°$.

This rotation should be the sum of the rotations of equimolar L-glutamic acid, L-aspartic acid and L-valine (leucine was racemic). So, 36.8 mg of L-glutamic acid (0.25 millimole), 33.3 mg of L-aspartic acid (0.25 millimole) and 29.3 mg of L-valine (0.25 millimole) were dissolved in 10 ml 1 N hydrochloric acid, and the observed rotation in 1 dm tube was +0.28°.

The Hydrazinolysis of Esperinic Acid and Esperin—The anhydrous hydrazine was prepared according to K. Kusama[9]. A solution of 5 mg of esperinic acid in 0.5 ml of anhydrous hydrazine was heated in a sealed tube at 100° for 10 hours. After evaporation of the hydrazine in a desiccator over sulfuric acid, the residue was dissolved in 2 ml of water. The dinitrophenylation was followed by the addition of 2 g of sodium bicarbonate and 12 ml of 5% dinitrofluorobenzene ethanol solution. After shaking for 2 hours, the reaction mixture was diluted with four times of water, acidified with 1 N hydrochloric acid, and extracted three times with ethylacetate using 30, 10 and 10 ml portions. The solvent layer was extracted six times with 2% sodium bicarbonate solution (each portion 12 ml). The aqueous layer was again acidified and extracted three times with ethylacetate. The ethylacetate extracts were evaporated in vacuo. After dinitrophenol was removed by sublimation, the residue was dissolved in acetone and spotted on paper (Tōyō filter paper No. 51). The paper chromatography was run with the mixture of *n*-butanol and 0.1% aqueous ammonium solution (1 : 1 V)[10]. The

24) J. F. Roland, Jr. and A. M. Gross, *Anal. Chem.*, **26**, 502 (1954).

two spots were observed (R_F 0.66 and 0.32). The R_F value 0.66 was coincided with that given by DNP-leucine under the same conditions. The substance of R_F 0.32 had reddish brown color in alkaline solution, so this substance was appeared to be

DNP-NH-CH-CONHNH-DNP or DNP-NH-CH-COOH[25].
$(CH_2)_{1-2}$ $(CH_2)_{1-2}$
COOH CONHNH-DNP

The hydrazinolysis of esperin gave the same results.

Reaction of Esperin and Esperinic Acid with Pyridine and Acetic Anhydride (Turner's method)—Five mg of esperin, 0.75 ml of acetic anhydride and 0.50 ml of pyridine were heated in a sealed tube at 150° for 2.5 hours. The contents of the tube were rinsed out with water and evaporated to dryness, and after repeating this process once more, the residue was hydrolyzed with 6 N hydrochloric acid. The hydrolyzate was chromatographed on paper with *n*-butanol, acetic acid and water mixture (4:1:2.5 V). The chromatogram developed with ninhydrin showed five spots, of which R_F values were 0.27, 0.33, 0.56, 0.70 and 0.75. On the same condition, the hydrolyzate of esperin showed four spots, of which R_F values were 0.27 (asp.), 0.33 (glu.), 0.56 (val.) and 0.70 (leu).

3-Amino-5-methyl-2-hexanone was prepared by the next method. Twenty mg of leucine, 1.0 ml of pyridine, 1.5 ml of acetic anhydride were boiled for 2 hours with reflux condenser. After the solvent was removed, the N-acetyl group was hydrolyzed by boiling in 6 N hydrochloric acid for 3 hours. The solution was concentrated under reduced pressure and the residue was dissolved in water and was spotted on paper. The paper chromatogram with above solvent system showed a spot at R_F 0.75, developed with ninhydrin.

Turner's reaction with esperinic acid gave the same result as that given by esperin.

In order to determine the configuration of C-terminal leucine, esperin (1 g) was reacted with acetic anhydride (60 ml) and pyridine (40 ml) at 150° for 3 hours in a sealed tube. The reaction product was hydrolyzed and, after decolorization with Darco G-60, leucine was isolated as described in the previous paper. Recrystallization from 70% ethanol gave 30 mg of plate crystal. $[\alpha]_D^{20}+6°$ (in 6 N HCl). The authentic sample of L-leucine showed $[\alpha]_D^{20}+13°$ (in 6 N HCl).

Enzymatic Reaction with Carboxypeptidase—The used carboxypeptidase was a recrystallized preparation. Esperinic acid (5 mg) was added to 10.7 ml of water and pH of the solution was adjusted to 7.6 by adding 0.3 ml of 0.1 N lithium hydroxide solution. To this solution, 1 ml of carboxypeptidase solution was added and was stood at 27°. Two ml of aliquots were taken at intervals, reacted with dinitrofluorobenzene and was assayed for DNP-amino acid by paper chromatography. After 24 hours no amino acid was liberated from esperinic acid.

Esperin was also unaffected with carboxypeptidase.

Partial Hydrolysis of Esperinic Acid—Esperinic acid (10 mg) was dissolved in 1.5 ml of glacial acetic acid and the same volume of 12 N HCl was subsequently added. The solution was stood in a sealed tube at 35° for 1 week. The solution was concentrated to dryness below 35° in vacuo and the residue was dried in a desiccator over NaOH and P_2O_5. The solid was extracted with 3 ml of water and water-soluble material was separated. The aqueous solution was concentrated to a small volume and was examined by paper chromatography. The chromatogram, which was run with *n*-butanol, acetic acid and water (4:1:2.5 V.) solvent system, showed the following spots, R_F 0.27 (asp.), 0.33 (glu.), 0.56 (val.), 0.68 (leu.), 0.75~0.85 (two or three peptides), 0.93 (peptide). The peptide, of which R_F value 0.93, was separated by the following method. The above solution was spotted on a sheet of paper at about 10 mm intervals, and two spots were placed 25 mm further away on both sides of the sheet. After running the chromatogram, both sides of the paper were cut off into the vertical strips which contained the material from the outer spots. These strips were dipped in the ninhydrin solution, and the resulting pattern served

25) K. Ohno reported that -CONHNH-DNP group had reddish brown color in alkaline solution while it became yellow in acidic solution, and this phenomenon could be applied to find -NH-CH-CO- and -NH-CH-COOH linkages, in polypeptides.
$(CH_2)_{1-2}$ $(CH_2)_{1-2}$
COOH CO
K. Ohno, *J. Biochem.* (Japan), **42**, 615 (1955).
S. Mizushima and S. Akaboai, Chemistry of Proteins (in Japanese) **4**, 253 (1956) (Kyoritu Pub.)

as a guide, which enabled the major portion of the chromatogram to be cut into horizontal strips. The peptide was eluted from the strips with water, and was hydrolyzed completely. When examined by paper chromatography, it showed the composition of aspartic acid, valine and leucine. The N-terminal residue of this pedtide was determined by DNP-method according to I.M. Lockhart and E.P. Abraham[26] and the paper chromatography of DNP-amino acid was done according to Gerhart Braunitzer[10]. The N-terminal residue of this peptide was determined as L-aspartic acid.

There were observed two or three spots of peptides at R_F 0.75~0.85 on the above chromatogram of partial hydrolyzate, but the separation of these peptides by the paper chromatography was appeared to be difficult. Therefore, the neutral peptides and neutral amino acids were separated from acidic peptides and acidic amino acids by ionic exchange resin according to E.P. Poponoe and Vincent du Vigneaud[27].

A solution of 50 mg of esperinic acid in 10 ml of a 1 : 1 mixture of glacial acetic acid and concentrated HCl was allowed to stand at 35° for 14 days in a sealed tube. It was concentrated to dryness in vacuo and the residue was stored in an evacuated desiccator over H_2SO_4 and NaOH pellets. The residue was extracted with 2 ml of water and the solution was diluted with water and passed through a column of Amberlite IR-4B (1 × 12 cm), which had been prepared as described by Consden, Gordon and Martin[28]. The solution was followed by water. The effluent and water contained neutral peptides and neutral amino acids. Then, the column was eluted with N HCl, which contained acidic peptides and acidic amino acids. Both fractions were examined by paper chromatography. The paper chromatogram of neutral fraction indicated the following ninhydrin-positive spots (solvent: *n*-butanol, acetic acid, H_2O, 4 : 1 : 2.5), R_F 0.56 (val.), 0.68 (leu.) and 0.85 (peptide). The acidic fractions showed the following spots, R_F 0.18 (peptide), 0.27 (asp.), 0.32 (glu.), 0.76 (peptide) and 0.86 (peptide). These peptides were separated by paper chromatography and their compositions were determined by the same way as described above.

The neutral peptide of R_F 0.85 was determined to be leucylleucine. The acidic peptide of R_F 0.18 contained glutamic acid and aspartic acid. The composition of acidic peptides of R_F 0.76 and 0.86 were both leucine, valine, glutamic acid and aspartic acid. It appeared the acidic peptide of R_F 0.76 contained one mole of leucine and the acidic peptide of R_F 0.86 contained two moles of leucine.

Curtius Degradation of Esperin Monohydrazide—Esperin (500 mg) was dissolved in 1.5 ml of hydrazine hydrate by adding several drops of ab. ethanol. After standing for 48 hours at room temperature, the separated needle crystals were recovered by filtration.

Yield: 260 mg. This compound was scarcely soluble in water and ethanol, and its infrared spectrum showed the absorption of –COOH group but no absorption corresponding to $-COO^-$. The recrystallized sample from aqueous methanol indicated m.p. 220°.

Anal. Calcd. for $C_{39}H_{71}N_7O_{11}$: N, 12.0. Found: N, 11.87.

This compound (10 mg) was dissolved in glacial acetic acid, and with cooling and stirring, 20 mg of sodium nitrite in water was added. This turbid solution, after adding 2 drops of 1:1 HCl, was warmed over a micro burner until foaming ceased, and then concd. HCl was added, and warming was continued till the precipitates completely dissolved. The solution was evaporated to dryness.

The resulting amorphous powder was hydrolyzed completely with concd. HCl. The paper chromatogram (solvent: *n*-butanol, acetic acid, H_2O; 4 : 1 : 2.5 V.) of this hydrolyzate showed ninhydrin-positive spots at R_F 0.70 (leucine), 0.56 (valine), 0.33 (glutamic acid) and 0.14, but the spot of aspartic acid (R_F 0.27) was disappeared. The amino acid of R_F 0.14 by the above solvent system gave R_F value 0.20 on the chromatogram developed with *t*-butanol, acetic acid acid and water (2:1:1 V.) mixture.

α,β-Diaminopropionic acid[29] showed R_F 0.20 by the latter solvent system.

Partial Hydrolysis of Esperin—Esperin (50 mg) was dissolved in glacial acetic acid and the same volume of concd. HCl was added. The solution was stood at 30° for 4 days. The solution was concentrated to dryness in vacuo and the residue was dried in a desiccator over NaOH and P_2O_5. The solid was extracted with ether and 26 mg of ether-soluble substances were separated. The ether-soluble substances were further hydrolyzed

26) I. M. Lockhart and E. P. Abraham, *Biochem. J.* **58**, 633 (1954).

27) E.A. Poponoe and Vincent du Vigneaud, *J. Biol. Chem.*, **206**, 353 (1954).

28) R. Consden, A.H. Gordon and A.J.P. Martin, *Biochem. J.*, **42**, 443 (1948).

29) α,β-Diaminopropionic acid could be obtained by the hydrolysis of viomycin. T. H. Haskell, S. A. Fusari, R. P. Frohardt and Q. R. Rartz, *J. Am. Chem. Soc.*, **74**, 599 (1952).

with 0.25 M barium hydroxide aqueous solution at 100° for 3 hours. After removing barium ion with oxalic acid, the solution was concentrated to a small volume and was examined by paper chromatography. The chromatogram showed one spot of aspartic acid.

In the parallel experiment, esperinic acid was hydrolyzed partially and the ether-soluble substances were hydrolyzed with barium hydroxide on the same condition as above. The chromatogram of this hydrolyzate showed no ninhydrin positive spot. And it was also determined that the peptide bonds of esperin and esperinic acid were rather strong and could not be hydrolyzed by boiling in 0.25 M barium hydroxide at 100° for 3 hours. These facts showed that esperin but not esperinic acid could produce an ester composed of the hydroxyfatty acid and aspartic acid by partial hydrolysis.

Acknowledgement The authors wish to express their gratitude to Dr. M. Shimizu and Mr. S. Ai of this company for their encouragement in performing this work, to Mr. Kusama, University of Tokyo, for his guidance in the experiment of hydrazinolysis. Special thanks are due to Mr. S. Inoue of this laboratory for his help in this research. The authors are indebted to the members of the Microanalytical Laboratories of the Department of Agricultural Chemistry of Tokyo University for the microanalysis and Mr. K. Aizawa, University of Tokyo, for the infrared absorption spectra.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 6, p. 547～551, 1959]

Studies on the Metabolic Products of *Macrosporium porri* Elliott
Part III. Structure of Macrosporin (Group II)

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Received April 3, 1959

Macrosporin (I), $C_{16}H_{12}O_5$, was ethylated with diazoethane to monoethylether (IV), which was oxidized to 4-methyl-5-ethoxy phthalic acid (V). Oxidation of monoethylether (IX) gave 3,5-dimethoxy phthalic anhydride (XI). From these results and I.R. absorption spectra of macrosporin and its derivatives, the structure of macrosporin is established to be 3,5-dihydroxy-7-methoxy-2-methyl anthraquinone.

As reported in the previous paper[1], macrosporins (I), $C_{16}H_{12}O_5$ was an anthraquinone derivative, having one methoxyl group at the β-position and two hydroxyl groups at the α and the β position respectively, and the monomethylether (II) had the methyl and methoxyl group at both β positions of the same ring. But it was not clear whether the methoxyl group ajacent to the methyl group existed originally in macrosporin or not. In order to solve this problem, the following experiments were carried out.

It is well known that the hydroxyl group at the α position of anthraquinone is hardly methylated with diazomethane but the one at the β position is easily. This general rule is also applicable to the ethylation with diazoethane. Macrosporin was ethylated with diazoethane to

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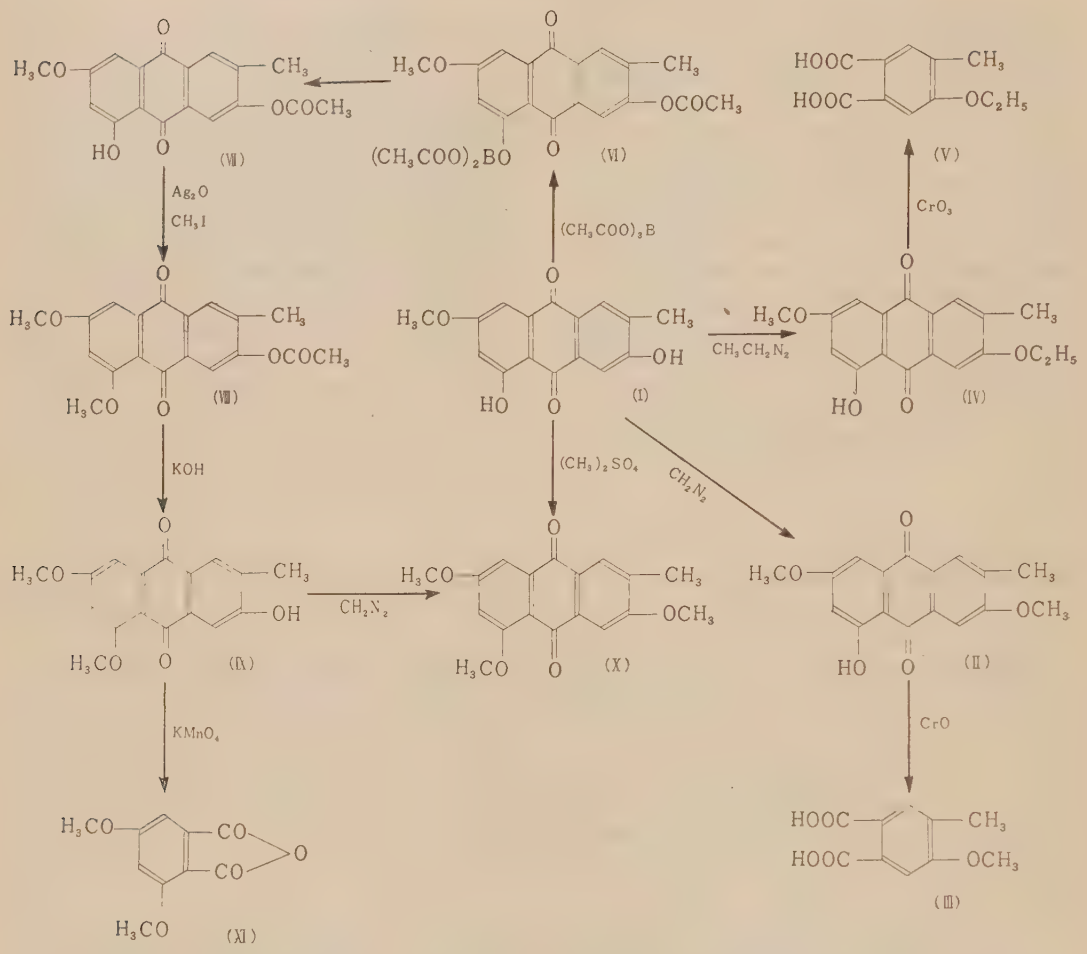
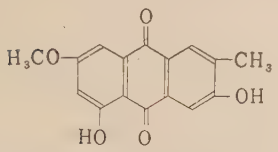
1) R. Suemitsu and M. Hiura, This Bulletin, **21**, 337 (1957).



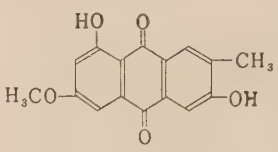
macrosporin monoethylether (IV), which was oxidized with chromic acid to the unknown colorless leaflets of m.p. 161°. This compound was proved to be identical with 4-methyl-5-ethoxy phthalic acid prepared from *o*-ethoxy-*p*-toluic acid. Consequently, macrosporin has the free hydroxyl group adjacent to the methyl group.

E. Jones and et al.[2] reported that the hydroxyl group at the β position of Rubiadin (1, 3-dihydroxy-2-methyl anthraquinone) was preferentially acetylated with boroacetic anhydride to 1-hydroxy-3-acetoxy-2-methyl anthraquinone. By the same method macrosporin monoacetate (VII) was obtained and methylated with methyl iodide and silver oxide to macrosporin monoacetyl monomethylether (VIII), which was hydrolysed with methanolic potassium hydroxide to macrosporin monomethylether (IX). The methylation of this monomethylether (IX) with diazomethane gave macrosporin dimethylether (X), which was also prepared by the exhaustive methylation of macrosporin with dimethylsulfate. Macrosporin monomethylether (IX) was oxidized with potassium permanganate to obtain colorless prisms which were purified by sublimation and melted at 149°. This substance was found to be iden-

2) E. Jones and A. Robertson, *J. Chem. Soc.*, **1930**, 1699.



(A)

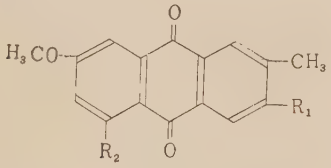


(B)

tical with 3, 5-dimethoxy phthalic anhydride (XI). From the above mentioned results, the formula of macrosporin should be either (A) or (B).

According to the research of anthraquinones reported by Tanaka[3], the hydroxyl or the methoxyl group substituted at the para position to the free carbonyl group displaces in dioxane solution the free carbonyl band by about 6~10 cm^{-1} towards lower frequencies than what normally assigned for anthraquinone (1676 cm^{-1}). Agreeing with this rule, macrosporin and its derivatives had the free carbonyl bands at 1661~1672 cm^{-1} in dioxane solution (Table I), while all of the related compounds of macrosporin which had no substituent at the para position to the free carbonyl group, such as methylpurpuroxantin[4] and its derivatives, were proved

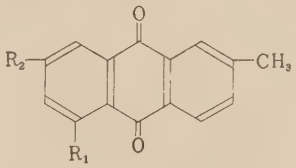
TABLE I. THE CARBONYL BAND OF MACROSPORIN AND ITS DERIVATIVES (in dioxane)



Compound		Free Carbonyl frequency (cm^{-1})	Chelated carbonyl frequency (cm^{-1})
R_1	R_2		
OH	OH	1669	1631
CH_3O	OH	1667	1631
CH_3O	CH_3COO	1667	—
CH_3COO	OH	1669	1630
CH_3COO	CH_3COO	1672	—
$C_6H_5CH_2O$	$C_6H_5CH_2O$	1661	—

3) O. Tanaka, *Pharm. Bull.*, **6**, 18 (1958).
4) A. Hutchison, *J. Chem. Soc.*, **1833**, 1142.

TABLE II. THE CARBONYL BAND OF METHYLPURPUROXANTIN AND ITS DERIVATIVES (in dioxane)



Compound		Free carbonyl frequency (cm^{-1})	Chelated carbonyl frequency (cm^{-1})
R_1	R_2		
OH	OH	1678	1634
OH	CH_3O	1678	1634
CH_3COO	CH_3O	1678	—

to have the free carbonyl bands at 1678 cm^{-1} (Table II). From these results, macrosporin was decided to have a para hydroxyl substituent to the free carbonyl group, and its structure was established to be 3, 5-dihydroxy-7-methoxy-2-methyl anthraquinone (A).

EXPERIMENT

Macrosporin Monoethylether (IV): Macrosporin (70 mg) was dissolved in the mixture of ether (130 ml) and acetone (200 ml) and an excess of an ethereal solution of diazoethane was added. After allowing it to stand overnight, the solvent was removed and a yellow residue was recrystallized from methanol to give fine yellow needles, m.p. 198~199°; yield 60 mg (78%). Found: C, 69.38; H, 5.38. Calcd. for $C_{18}H_{16}O_5$: C, 69.22; H, 5.16% (chelated carbonyl band, 1620 cm^{-1}; non-chelated carbonyl band, 1653 cm^{-1} in nujol).

Oxidation of Macrosporin Monoethylether (IV): Macrosporin monoethylether (100 mg) was dissolved in the mixture of glacial acetic acid (20 ml) and acetic anhydride (5 ml), and to this mixture of chromium trioxide (220 mg) in water (two drops) and glacial acetic acid (20 ml) was gradually added with continuous shaking for twenty minutes with heating on the boiling water-

bath. Heating and stirring were continued for more fifteen minutes. The dark green reaction mixture was cooled, evaporated in vacuo to a syrup, which was acidified with hydrochloric acid and extracted with ether. The ethereal solution was shaken with 5% sodium carbonate solution (400 ml). The alkaline solution was acidified with hydrochloric acid and extracted with ether, and on evaporation crude crystals were obtained. This material was recrystallized from water to give rise to colorless leaflets, m.p. 161°; yield 10 mg (14%). This sample was found to be identical with the authentic specimen of 4-methyl-5-ethoxy phthalic acid, m.p. 160°, by both the mixed melting point test and infra-red absorption spectra.

Synthesis of 5-methyl-6-ethoxy phthalide: *o*-Ethoxy-*p*-toluic acid (400 mg) was heated with formaline (1.6 ml) and concd. hydrochloric acid (2 ml) on the boiling water-bath for six hours. The crystals separated were filtered and recrystallized from methanol to give rise to colorless needles, m.p. 114~115°; yield 200 mg (47%). Found: C, 69.19; H, 6.47. Calcd. for $C_{11}H_{12}O_3$; C, 68.76; H, 6.29%.

4-Methyl-5-ethoxy phthalic acid from 5-methyl-6-ethoxy phthalide:

5-Methyl-6-ethoxy phthalide (600 mg) in 10% potassium hydroxide solution (15 ml) was gradually treated with powdered potassium permanganate (670 mg) under cooling and allowed to stand overnight at room temperature. The reaction mixture was acidified and extracted with ether. Evaporation of ether left the crude matter, which was recrystallized from water to give rise to crystals of m.p. 140~150°; yield 330 mg (47%). The substance (200 mg) was refluxed with acetic anhydride for one hour. The solvent was removed in vacuo and the residue was recrystallized from *n*-hexane to give white needles of 4-methyl-5-ethoxy phthalic anhydride, m.p. 143~144°; yield 100 mg. The substance (100 mg) was dissolved in hot water (5 ml) and boiled for five minutes. The crude acid separated was recrystallized from water to give rise to colorless needles, m.p. 160°; yield 60 mg (55%). Found: C, 59.22; H, 5.61. Calcd. for $C_{11}H_{12}O_5$; C, 58.92; H, 5.40%.

Macrosporin Monoacetate (VII): Macrosporin (200 mg) in acetic anhydride (10 ml) was refluxed with boroacetic anhydride for five minutes. The removal of the solvent in vacuo gave the yellow matter to which water (50 ml) was added and stood overnight. The yellow crystals separated were recrystallized from acetone to give rise to slender orange needles, m.p. 203~204°; yield 150 mg (65%). Found: C, 66.34; H, 4.24. Calcd. for $C_{18}H_{14}O_8$: C, 66.25, H, 4.32% (carbonyl band of acetate, 1741 cm^{-1}; chelated carbonyl band, 1626 cm^{-1}; non-chelated carbonyl band, 1667 cm^{-1} in nujol).

Macrosporin Monoacetyl-monomethylether (VIII): Macrosporin monoacetate (600 mg) in acetone (40 ml) was refluxed with silver oxide (4 g) and methyl iodide (3 ml) for five hours. The hot solution was filtered and evaporated to a solid which was recrystallized from acetone to give yellow needles, m.p. 228~229°; yield 400 mg (64%). Found: C, 67.05; H, 4.94. Calcd. for $C_{19}H_{16}O_6$: C, 67.05; H, 4.75% (carbonyl band of acetate, 1745 cm^{-1}; non-chelated carbonyl band, 1664 cm^{-1}, 1650 cm^{-1} in nujol).

Macrosporin Monomethylether (IX): Macrosporin monoacetyl monomethylether (150 mg) was dissolved in 5% methanolic potassium hydroxide solution, kept at room temperature for 2.5 hours and then acidified with hydrochloric acid. After filtration, the crude matter was recrystallized from acetone to give rise to yellow needles, m.p. 295~297° (dec.); yield 100 mg (71%). Found: C, 68.33; H, 4.75: Calcd. for $C_{17}H_{14}O_5$: C, 68.45; H, 4.73% (non-chelated carbonyl band, 1656 cm^{-1} in nujol).

Macrosporin Dimethylether (X): Macrosporin monomethylether (50 mg) was dissolved in the mixture of acetone (100 ml) and ether (50 ml) and an excess of an ethereal solution of diazomethane was added. And it was allowed to stand overnight, the solvent was removed to give rise to yellow needles, which was recrystallized from acetone to give fine yellow needles, m.p. 260°; yield 30 mg (58%). This sample was found to be identical with macrosporin dimethylether (X), m.p. 260° by both the melting point test and infra-red absorption spectra.

Oxidation of Macrosporin Monomethylether (IX): Macrosporin monomethylether (300 mg) was dissolved in 2N sodium hydroxide solution and 3% potassium permanganate solution (60 ml) was added with constant shaking for thirty minutes with heating on the boiling water-bath. Heating and stirring were continued for another one hour. The reaction mixture was filtered, acidified with hydrochloric acid and extracted with ether. The removal of ether gave a crude solid. Sublimation in high vacuum followed by recrystallization from *n*-hexane gave colorless needles, m.p. 149°; yield 10 mg (5%). This sample was found to be identical with the authentic specimen of 3,5-dimethoxy phthalic anhydride[5], m.p. 149°, by both the mixed melting point

5) G. Graves, *J. Am. Chem. Soc.*, **45**, 2439 (1923).

test and infra-red absorption spectra. Found : C, 57.50; H, 3.82. Calcd. for $C_{10}H_8O_5$: C, 57.69; H, 3.87%.

Acknowledgement We are indebted to Prof. S. Takei of Kyoto University for his helpful advice. Grateful thanks are also due to Prof. T. Mitsui of Kyoto University for the elementary analysis, to Mr. O. Tanaka of Tokyo University for his valuable suggestions, and to Mr. Y. Matsui of Rakuno College for supplying sample.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 6, p. 551~554, 1959]

Studies on Browning Reactions between Sugars and Amino Compounds

Part IV. Identification of 5-Hydroxymethylfurfural formed from Aromatic Amine-N-Hexosides and its Role in the Browning Reaction*

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Received April 6, 1959

N-D-Glucosyl-*p*-aminobenzoic acid has been found to form melanoidins in methanol solution acidified with hydrogen chloride at 25°. From the reaction mixture 5-hydroxymethyl-furfural (HMF) has been isolated and identified as its 2,4-dinitrophenylhydrazone. So, comparisons between the browning reaction of HMF or furfural with aromatic amine and that of the corresponding N-glycosides have been made under the same condition. From the results obtained, it has been shown that, under the described condition, furfural is almost inactive for browning, while on the contrary, HMF is active and plays an important role in the browning reaction.

In the previous paper of this series[1], it has been found that aromatic amine-N-xylosides produce furfural, melanoidins and red pigments in the methanol solution acidified with hydrogen chloride at room temperature. In this paper, the browning reaction of aromatic amine-N-hexoside is investigated under the same condition.

N-D-Glucosyl-*p*-aminobenzoic acid (N-D-glucosyl-PABA) in methanol suspension acidified with hydrogen chloride at 25° gradually dissolves along with the formation of brown color. The spectrum of this colored solution indicates that melanoidin formation has occurred and that red pigment formation characteristic in the case of aromatic amine-N-pentoside described in the previous paper[1] is almost negligible as shown in Fig. 1. From the reaction mixture of N-D-glucosyl-PABA in methanol solution acidified with hydrogen chloride at 25° for seventy-two hours, HMF has been isolated as its 2, 4-dinitrophenylhydrazone by adsorption chromatographies on alumina and silicic acid. Further, N-D-galactosyl-

* Presented at the Symposium entitled "Amino-Carbonyl Reaction Connected with Foodstuffs" of the Annual Meeting of the Agricultural Chemical Society of Japan, Tokyo, April 8, 1959.

1) Part III: This Bulletin, **22**, 85 (1958).

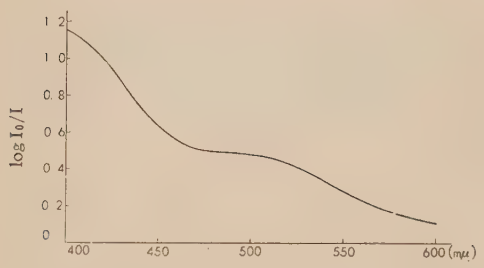


FIG. 1. Browning of N-D-Glucosyl-PABA.

The solution (0.5M) of N-D-glucosyl-PABA in methanolic hydrogen chloride (0.5N) was stood at 30° for twenty-four hours and diluted to 0.001M.

PABA in methanol solution acidified with hydrogen chloride at 25° for twenty-four hours has afforded 2, 4-dinitrophenylhydrazone of HMF in 7.5% of the theoretical yield as a mixture of syn- and anti-forms.

Concerning the 2, 4-dinitrophenylhydrazone of HMF, several melting points have been reported. These are 184° (cor.)[2], 197~199° (cor.)[3] and 198~200° (cor.)[3,4]. The discrepancy of these melting points depends upon syn- and anti-isomerism[4~6]. 2, 4-Dinitrophenylhydrazone prepared from pure HMF (m.p. 34°) showed m.p. 221° (uncor.), when it was recrystallized from benzene. The ultraviolet and visible absorption spectrum of this hydrazone (Fig. 2)

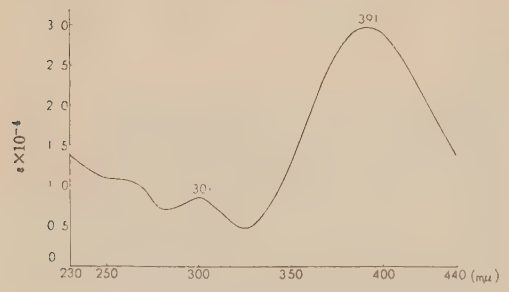


FIG. 2. Anti-form of HMF 2,4-Dinitrophenylhydrazone (in Methanol).

indicates that its configuration is anti-form, because of the similarity with the spectrum of anti-form of 2, 4-dinitrophenylhydrazone of furfural[1]. 2, 4-Dinitrophenylhydrazone of HMF obtained from N-glycoside by the procedure described above raised its melting point to 218° through thrice recrystallizations from benzene. These facts indicate that pure anti-form melts at 221°, but the mixture of syn- and anti-forms melts at ca. 180°. In this paper, attempts to prepare a pure syn-form have resulted in failure.

As described above, aromatic amine-N-glycosides form furfural or HMF and melanoidins in methanolic hydrogen chloride at room temperature. So, the role of furfural or HMF in melanoidin formation of N-glycosides under the condition described above has been investigated (Tables I and II).

TABLE I. INFLUENCE OF FURFURAL TO BROWNING OF AROMATIC AMINE-N-XYLOSIDES.

A: Each 0.2M methanol solution acidified to 0.2N with hydrogen chloride was stood at 27° for twenty-four hours.
B: Each 0.5M methanol solution acidified to 0.5N with hydrogen chloride was stood at 27° for twenty-four hours.
All of them were diluted to 0.002M solution.

		Reacting substance	$\log I_0/I$ at 600 mμ
A.	1.	Furfural+PABA	0.021
	2.	N-D-Xylosyl-PABA	0.156
	3.	N-D-Xylosyl-PABA+furfural	0.208
	4.	N-D-Xylosyl-PABA+PABA-HCl	0.420
	5.	N-D-Xylosyl-PABA+PABA-HCl +furfural	0.405
B.	1.	Furfural+aniline	0.028
	2.	N-D-Xylosyl-aniline	0.184
	3.	N-D-Xylosyl-aniline+furfural	0.121
	4.	N-D-Xylosyl-aniline+aniline-HCl	0.524
	5.	N-D-Xylosyl-aniline+aniline-HCl +furfural	0.377

In the case of furfural, as shown in Table I, the effect of addition of furfural to N-xyloside is almost negligible, because furfural reacts with aromatic amine hydrochloride which has been added or formed by degradation of N-xyloside to produce a red pigment[1] rather than to produce melanoidin. The addition of aromatic amine hydrochloride to N-xyloside with or with-

2) J. J. Blanksma and M. L. Wackers, *Rec. trav. chim.*, **55**, 658 (1936).
3) B. L. Scallet and J. H. Gardner, *J. Am. Chem. Soc.*, **67**, 1934 (1945).
4) A. Wahhab, *ibid.*, **70**, 3580 (1948).
5) H. Bredereck, *Ber.*, **65**, 1833 (1932).
6) L. I. Braddock, et al., *Anal. Chem.*, **25**, 301 (1953).

TABLE II. EFFECT OF HMF TO BROWNING OF N-D-GLUCOSYL-PABA

Each 0.2M methanol solution acidified to 0.2N with hydrogen chloride was stood at 27° for twenty-four hours. All of them were diluted to 0.002M solution.

Reacting substance	$\log I_0/I$ at 600 mμ
1. HMF+PABA	0.022
2. N-D-Glucosyl-PABA	0.078
3. N-D-Glucosyl-PABA+HMF	0.083
4. N-D-Glucosyl-PABA+PABA	0.003
5. N-D-Glucosyl-PABA+PABA+HMF	0.159
6. N-D-Glucosyl-PABA+PABA-HCl	0.126
7. N-D-Glucosyl-PABA+PABA-HCl HMF	0.273

out furfural shows a remarkable effect for melanoidin formation (Table I). These facts lead to the conclusion that there is another active compound except furfural which is formed by degradation of N-xyloside and reacts with aromatic amine hydrochloride to produce melanoidin.

In the case of HMF, as shown in Tabble II, N-D-glucosyl-PABA is used in place of N-xylosides and the addition of HMF to N-D-glucosyl-PABA shows a considerable effect on melanoidin formation. The addition of PABA hydrochloride to N-D-glucosyl-PABA with or without HMF shows a stronger effect. The addition of free PABA instead of PABA hydrochloride remarkably decreases melanoidin formation (Table II). This fact shows that the active form of N-glucoside is N-D-glucosyl-PABA hydrochloride, because the addition of free PABA deprives hydrogen chloride of N-D-glucosyl-PABA hydrochloride which is rather more unstable than its free form in accordance with the observation of Rosen et al.[7].

Concerning the browning of furfural, an analogue of HMF, Dunlop, Stout and Swadesh[8] have shown it to be oxygen dependent. And further, Rosen, Johnson and Pigman[7] who investigated the darkening of N-D-glucosylaniline, discussed the role of HMF suggesting that the browning of HMF may be oxygen dependent. However, according to the results shown in Table II, it is difficult to conceive that the effect of HMF is solely dependent on oxygen. It seems that there is an interaction between HMF and N-D-glucosyl-PABA or its decomposed product. But it is not to be considered that all of the origin of melanoidin formed from N-D-glucosyl-PABA depends upon HMF alone, but it is rather naturally cosidered that there is another intermediate except HMF quite the same as furfural in the case of N-pentoside.

EXPERIMENTAL

All melting points except those indicated were uncorrected. 2,4-Dinitrophenylhydrazine (2,4-DPH) solution was prepared by Shriner's method.[9] Spectrophotometric measurements were carried out with a Hitachi Photo-Electric Spectrophotometer in methanol solution. Infrared spectra were measured by a Kōken DS-301 Infrared Spectrophotometer in Nujol mull.

Preparation of N-D-Glucosyl-PABA.[10] A quantity of 6.4g of finely powdered D-glucose and 5.0g of PABA were added to 20 ml of 94% ethanol, and heated at 80~85° in a water bath with stirring for thirty minutes. After heating, to the reacting solution, 3 ml of water was added and allowed to stand at room temperature and thereafter in a refrigerator overnight. The crystal was filtered with suction, washed with 94% ethanol and dried in a desiccator with calcium chloride at room temperature. The yield was 7.6 g of needles (68%, m.p. 129~130°, dec.). Recrystallization from 50% methanol gave 5.1 g, m.p. 132° (dec.). *Anal.* Calcd. for $C_{13}H_{17}NO_7 \cdot H_2O$: C, 49.21; H, 5.99; N, 4.42%. Found: C, 50.10; H, 5.96; N, 4.42%.

When this monohydrated crystal was washed with a mixture of acetone and methanol (2:1 by volume) repeatedly, its melting point changed to 151° (dec.). This crystal (m.p. 151°, dec.) was restored to the monohydrated crystal (m.p. 132°, dec.) by recrystallization from 50% methanol. Accordingly, the crystal of m.p. 151° (dec.) is anhydrous.

Preparation of N-D-Galactosyl-PABA.[10] A quantity of 12.8 g of finely powdered D-galactose and 10 g of

7) L. Rosen, K. C. Johnson and W. Pigman, *J. Am. Chem. Soc.*, **75**, 3460 (1953).
8) A. P. Dunlop, P. R. Stout and S. Swadesh, *Ind. Eng. Chem.*, **38**, 705 (1946).
9) R. L. Shriner and R. C. Fuson, "The Systematic Identification of Organic Compounds", p. 171 (1948).
10) Y. Inouye, K. Onodera and S. Kitaoka, *J. Agr. Chem. Soc. Japan*, **25**, 59 (1951-52).

PABA were added to 40 ml of 94% ethanol, and heated at 80~85° in a water bath with stirring for thirty minutes. D-Galactose dissolved gradually, and the solution became orange. After heating, a small amount of undissolved material was removed by decantation, and the clear solution was furnished with 3 ml of water and held at room temperature, and crystallization occurred. After crystallization was completed, it was kept in a refrigerator overnight. The crystal was filtered with suction and washed with 94% ethanol and dried in a desiccator with calcium chloride. The yield was 17.3 g of needles, m.p. 148~150° (dec.). Recrystallization from 80% methanol gave 13.3 g of needles, m.p. 157~158° (dec.) (58% of the theory). *Anal.* Calcd. for $C_{13}H_{17}NO_7 \cdot H_2O$: C, 49.21; H, 5.99; N, 4.42%. Found: C, 50.01; H, 5.92; N, 4.23%.

Preparation of 2,4-Dinitrophenylhydrazone of HMF. HMF was prepared by Haworth's method[11]. This HMF melted at 34° and its semicarbazone gave m.p. 193° (dec.) and the following analytical data. *Anal.* Calcd. for $C_7H_9N_3O_3$: C, 45.90; H, 4.95; N, 22.94%. Found: C, 45.74; H, 4.92; N, 22.99%.

2,4-Dinitrophenylhydrazone of HMF was prepared in the following manner. The methanol solution of HMF was added to 2,4-DPH solution[9] and the resulting precipitate was filtered with suction, washed with 50% ethanol repeatedly and dried on sulfuric acid. This was twice recrystallized from hot benzene. Red needles were obtained, m.p. 221°. *Anal.* Calcd. for $C_{12}H_{10}N_4O_6$: C, 47.06; H, 3.29; N, 18.30%. Found: C, 47.22; H, 3.47; N, 17.90%.

Identification of HMF Formed from N-D-Glucosyl-PABA or N-D-Galactosyl-PABA in Methanolic Hydrogen Chloride. A quantity of 12.7 g of N-D-glucosyl-PABA suspended in 80 ml of methanolic hydrogen chloride (0.5 N) was allowed to stand at 25° for seventy-two hours. N-D-Glucosyl-PABA gradually dissolved and the reacting solution became deep brown. To this solution, 2,4-DPH (2.0 g) solution[9] was added and then 50 ml of water were slowly added and the resulting mixture was held at room temperature for two hours. The precipitate was filtered, washed with 50% ethanol and dried. Then it was extracted with ethylacetate and filtered. The filtrate was passed through a column of alumina (80 g, Brockmann) packed with ethylacetate to remove the fraction adsorbed with alumina in ethylacetate. The eluate was dried up and dissolved in benzene and charged to adsorb with a column (diameter, 2 cm) of a mixture of silicic acid (16 g, Mallinckrodt) and hyflosupercel (8 g, JohnsManville-Wakō) (2 : 1 by weight) packed with benzene. After feeding, the column was washed with benzene and then eluted with benzene containing ethylacetate up to 20% by volume. The eluate from the first orange band was discarded and that from the following main red band was collected and concentrated to 10 ml under reduced pressure. The concentrate was allowed to stand at room temperature for one day, filtered off, washed with benzene and dried. This powder was dissolved in benzene again and the solution was charged to adsorb with a column (diameter, 2 cm) of alumina (40 g) packed with benzene. After feeding, the column was washed with benzene and eluted with benzene containing ethylacetate up to 30% by volume. The elutate was then dried up. The yield was 415 mg, m.p. ca. 180°, of orange crystalline powder. The powder was a mixture consisting of syn- and anti-forms of 2,4-dinitrophenylhydrazones of HMF. One recrystallization from 95% ethanol and three recrystallizations from benzene gave red needles of pure anti-form, m.p. 218°. Upon admixture with an authentic sample (m.p. 221°) described above, it melted at 219° and its infrared spectrum was identical with that of the authentic sample.

A quantity of 12.7 g of N-D-galactosyl-PABA suspended in 80 ml of methanolic hydrogen chloride (0.5N) was allowed to stand at 25° for twenty-four hours. The resulting solution was treated similarly as described above. The yield of the mixture of syn- and anti-forms of HMF 2,4-dinitrophenylhydrazone was 915 mg, m.p. ca. 175°. Yield was 7.5% of the theory.

Acknowledgements The author wishes to express his sincere gratitude to Prof. Yusuke Sumiki for his constant guidance and encouragement throughout the course of this work. Indebts are also due to Mr. K. Aizawa for the infrared spectrophotometric measurements, and Mrs. Sato, Miss Isobe and Miss Suzuki for the micro elementary analyses.

11) W. W. Haworth and W. G. M. Jones, *J. Chem. Soc.*, **1944**, 667.

Possibility that Myrosinase is a Single Enzyme and Mechanism of Decomposition of Mustard Oil Glucoside by Myrosinase

Sir:

The mustard oil glucoside is split into mustard oil, glucose and potassium bisulfate by myrosinase. Neuberg[1] suggested that myrosinase was a mixture of two enzymes, myrosulfatase and thioglucosidase, which reacted separately on the glucoside, and myrosulfatase broke sulfuric acid ester-linkage and thioglucosidase hydrolized thioglucoside-linkage. This view has been widely accepted.

We have carried out experiments on myrosinase and obtained the following results which lead us to a different conclusion from that of Neuberg.

(1) All attempts to separate the two enzymes of myrosinase of white mustard seed by means of fractional precipitation, electrophoresis and other methods were unsuccessful, and the ratio of the one activity to the other in each fraction was always one to one.

(2) Neuberg described that the ratio of the two activities changed when myrosinase were treated with kaolin or mercuric acetate. We have traced their experiment but no change of activity ratio has been observed. (There are some doubts about Neuberg's method of determining the thioglucosidase activity.)

(3) Myrosinase was extracted from white mustard seed, and purified approximately 200-fold. The activity ratio of the two action of this purified sample and that of each fraction in the process of purification has been always one.

(4) We have determined the optimum pH, temperature and others of the purified sample and found that these values of myrosulfatase action have been the same as those of thioglucosidase action.

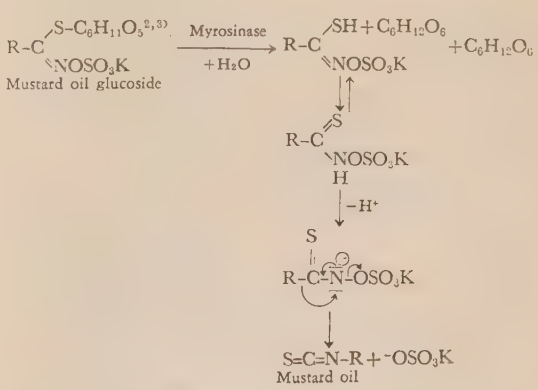
(5) Inhibition experiments showed that myrosinase was inhibited by SH-inhibitor and remarkably activated by ascorbic acid. Both the inhibition and the activation of myrosulfatase parallelled with those of thioglucosidase. No inhibitor has hindered one enzyme in preference to the other.

(6) We have attempted to inactivate enzymes by means of heating, irradiations of γ-ray and other methods. These effects were always parallel in both enzyme-actions.

(7) The activity ratios of the two actions of myrosinase prepared from various kinds of crucifers were determined to be one without any exception.

These results show a strong possibility that myrosinase is a single enzyme.

Our foregoing experimental results can be readily understood by the following reaction mechanism. The first enzymatic step is the hydrolysis of the thioglucoside linkage of the glucoside followed by the spontaneous removal



(1) C. Neuberg and O. Schoenbeck, *Biochem. Z.*, **265**, 223 (1933).

(2) M. G. Ettlinger and A. J. Lundeen, *J. Am. Chem. Soc.*, **78**, 4172 (1956).

(3) M. G. Ettlinger and A. J. Lundeen, *ibid.*, **79**, 1764 (1957).

of the sulfate. This mechanism is supported not only from the analogous observation by Hurd[4] on the rearrangement of hydroxamic acid but also from the views of electronic theory as shown in the figure above.

Since Neuberg had described that myrosinase has been a mixture of myrosulfatase and thioglucosidase, myrosinase became an obsolete word but in view of our idea, we think that the word myrosinase should be used again. The enzyme must be a kind of glucosidase and may not be any sulfatase in spite of the fact that the enzyme breaks the sulfuric acid ester-linkage indirectly.

(The detailed description of these experiments and the discussion will appear in the Journal of the Agricultural Chemical Society of Japan.)

We wish to express our sincerest thanks to Prof. Y. Sumiki and Prof. B. Maruo of the University of Tokyo for their advice and encouragement.

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(4) C.D. Hurd and L. Bauer, *J. Am. Chem. Soc.*, **76**, 2791 (1954).

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 6, p. 556~557, 1959]

Neocycasin B and Macrozamin*

(Studies on Some New Azoxy Glycosides of *Cycas revoluta* Thunb. Part II)

Sir:

In the previous paper[1], we have reported on neocycasin A, one of the series of aliphatic azoxy glycosides, found in the seeds of Japanese cycad together with cycasin[2]: In the carbon column chromatography of the extracts of cycad seeds, the spot A group, assumed to be azoxy glycosides, were detected by paper chromatography in the 10 and 40% ethanolic eluates. The major component in the latter fraction, neocycasin A, was crystallized and concluded to be β-laminaribiosyloxyazoxymethane.

In the present communication, the two azoxy glycosides in the former 10% ethanolic eluate are described. The one, visualized as spot A_4, was proved to be β-gentiobiosyloxyazoxymethane and was named here neocycasin B. The other, A_3, was identified with macrozamin which was first isolated from Australian cycad[3,4].

The fractionation by gradient elution[5] from the carbon column resulted in less efficiency, thereby, the two azoxy glycosides in company with oligosaccharide were eluted in the range of 40 to 45% concentration of methanol. Then, this mixture was rechromatographed on a column of powdered cellulose (80 g; dia. 17×550 mm) by using a mixture of butanol-ethanol-water (5:2:1) as a successful solvent, and the fractions (10 ml) were collected and checked by paper chromatography. The fractions No. 55~70 and 82~120 were found to be homogeneous as for spot A_3 and A_4, respectively. From the latter fraction, which was handled for crystallization unsuccessfully, 150 mg of neocycasin B was obtained as hygroscopic amorphous powder.

Neocycasin B thus obtained was decomposed

* Presented at the meeting of the Agricultural Chemical Society of Japan held in Tokyo, April 9, 1959.

1) K. Nishida, A. Kobayashi, T. Nagahama and T. Numata, This Bulletin, **23**, 460 (1959).

2) K. Nishida, A. Kobayashi and T. Nagahama, *ibid.*, **19**, 77 (1955).

3) J. M. Cooper, *Proc. Roy. Soc. N.S.W.*, **74**, 450 (1940); *C.A.*, **35**, 4917 (1941).

4) B. W. Langley, B. Lythgoe and N. V. Riggs, *J. Chem. Soc.*, **1951**, 2309.

5) R. M. Bock and Nan-Sing Ling, *Anal. Chem.*, **26**, 1543 (1954).

with alkali to give cyan, and displayed in its ultraviolet spectrum an absorption peak at 215 mμ ($\log \varepsilon_{max}=3.81$) and an inflection at 275 mμ ($\log \varepsilon_{infl}=1.56$), which are characteristic of aliphatic azoxy group.

The glycoside show $[\alpha]_D^{18}=-37.6°$ (c 1.0 in water). Acetylation gave its acetylate in long prisms (FIG. 1), m.p. 173°~174°, $[\alpha]_D^{20}=-29.4°$ (c 0.3 in $CHCl_3$). *Anal.* Found: C, 46.04; H, 5.70; N, 4.06%. Calcd. for $C_{14}H_{19}O_{12}N_2\cdot(CH_3CO)_7\cdot H_2O$: C, 46.28; H, 5.83; N, 3.86%. MW, Found: 705 (Rast), Calcd.: 726. CH_3CO, Found: 7.07 mols. per mol. Deacetylation of the acetylate was followed to a recovery of the original glycoside.

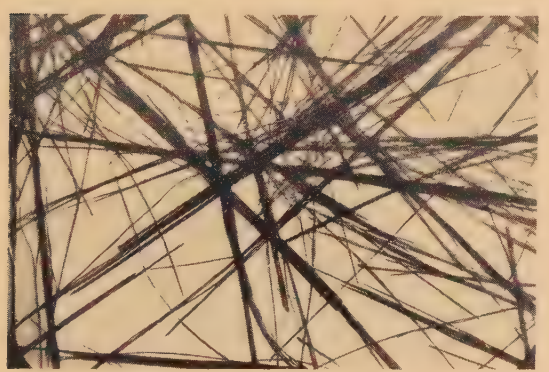

FIG. 1. Microphotograph of Heptaacetylneocycasin B

By complete acid hydrolysis (1N HCl, 2 hrs., 100°), neocycasin B liberated glucose, which was identified paper chromatographically and as phenylglucosazone. Its amount was equivalent to 1.94 mols. per mol. of neocycasin B, where it was assumed to be glucosylcycasin. The presence of formaldehyde which was derived from the aglycone, in the above hydrolysate, was proved as its dimedone derivative. Furthermore, the partial acid hydrolysate (0.2N H_2SO_4, 1 hr., 100°) being examined, the spots of cycasin, glucose and one corresponding to gentiobiose or isomaltose were detected.

Since neocycasin B was found to be attacked by emulsin at both holosidic and heterosidic linkages, it must be β-gentiobioside. It was further confirmed by following data.

The glycoside was treated with 50% acetic acid and zinc-copper couple. The reaction mixture, in which single spot corresponding to the biose mentioned above was found, was filtered and evaporated to dryness, and the residue was acetylated with pyridine and acetic anhydride. The resultant acetylate, crystallized from ethanol, m.p. 187°~188°, was octaacetyl-α-gentiobiose.

Accordingly, it is concluded that neocycasin B is β-gentiobiosyl-oxyazoxymethane, i.e. 6-0-β-D-glucosylcycasin.

On the other hand, the syrup from the said fraction No. 55~70 was subjected to identify as for macrozamin. The R_F value of spot A_3 in the above fraction was in close agreement with that of authentic macrozamin, and, in the partial hydrolysate, the spots of cycasin, xylose and glucose were recognized certainly. From these facts including the characteristic absorption in ultraviolet spectrum, it might be now almost clear, though not hitherto, that a little of macrozamin, 6-O-β-D-xylosylcycasin, also, exists in Japanese cycad.

We are indebted to Dr. N. V. Riggs of New England University, N.S.W., Australia, for kindly supplying the pure crystals of macrozamin.

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Received August 4, 1959

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 6, p. 558~559, 1959]

The Glyoxylic Reductase-Glycollic Oxidase System in Microorganism*

Sir:

The bacteria of *coli-aerogenes* have already been found to contain isocitritase, the enzyme which catalyzes the degradation of isocitrate into glyoxylate and succinate[1~4]. It has furthermore been observed that the cell-free extracts of these organisms possess a very poor ability to produce α-ketoglutarate from citrate, but the addition of TPN** to the cell-free extracts brings about an increase in the yield of α-ketoglutarate even under the anaerobic conditions[4]. In the present investigation, the authors' interest has been directed to the mode of the anaerobic degradation of citrate to α-ketoglutarate. When the cell-free extracts of *coli-aerogenes* were anaerobically incubated with citrate in the presence of a catalytic amount of TPN, glyoxylate was decreasing against increasing yield of α-ketoglutarate. Chromatographic analysis[5,6] showed that glycollic and succinic acids in addition to α-ketoglutaric acid were formed in the presence of both citrate and TPN. Glycollic acid was also identified by a colour reaction[7] after being eluted from the paper chromatograms. It has now been demonstrated that the anaerobic formation of α-ketoglutarate is the result of a coupling reaction between isocitric acid dehydrogenase and glyoxylic acid reductase. FIG. 1 shows the oxidation of TPNH or DPNH by glyoxlate in the presence of bacterial glyoxylic acid reductase. The rate of reaction with DPNH was observed to be about half that with TPNH.

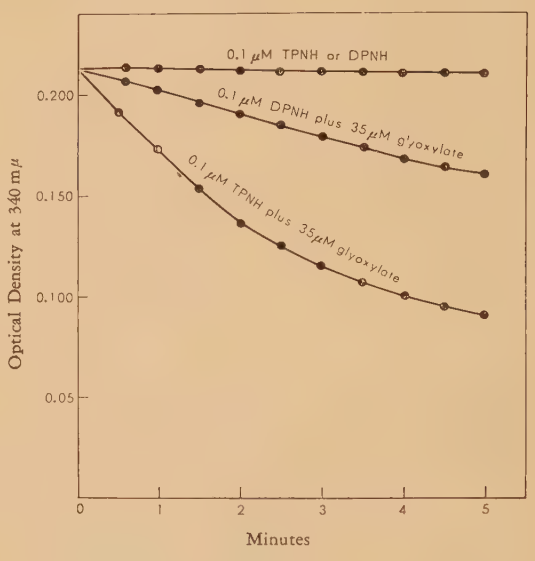

FIG. 1. Oxidation of TPNH and DPNH by Glyoxylate in Presence of Glyoxylic Acid Reductase of *A. aerogenes*

The reaction mixtures contained 250 μM phosphate buffer, pH 6.4, approx. 0.1 μM TPNH or DPNH, and 1 ml of the ammonium sulfate 0.4~0.7 saturation fraction (obtained from 150 mg cells of strain B-2 of *A. aerogenes* grown aerobically at 30°C for 20 hours on 1.5% Na-citrate plus 1% bouillon); the indicated amount of Na-glyoxlate was added at zero time; total volume, 3.0 ml. Oxidation of pyridine nucleotides was determined at room temperature spectrophotometrically at 340 mμ with a Beckman DU spectrophotometer, using 3 ml quartz cells, 1 cm light path.

Glyoxylic acid reductase has been found in *E. coli*, *A. aerogenes* and in *Ps. fluorescens*.

On the other hand, the authors have found that the oxidation of glycollate to glyoxylate by molecular oxygen is catalyzed by the dried cells of *coli-aerogenes* bacteria and baker's yeast. The glycollic acid oxidase of *coli-aerogenes* (*E. coli* and *A. aerogenes*) was isolated and partially purified by ammonium sulfate fractionation. The oxidase was resolved to yield an inactive apoenzyme by a modified procedure of Warburg and Christian[8]. The reactivation of this apoenzyme by flavin nucleotides is shown in Table I. The

* A part of this paper was read before the Meeting of the Kansai Division of the Agricultural Chemical Society of Japan held in Kyoto, April 25, 1959.

** The following abbreviations have been used: TPN=triphosphopyridine nucleotide; DPNH and TPNH=reduced di- and triphosphopyridine nucleotides.

1) R. A. Smith and I. C. Gunsalus, *Nature*, **175**, 774 (1955).
2) D. T. O. Wong and S. J. Ajl, *Nature*, **176**, 970 (1955).
3) H. Katagiri and T. Tochikura, This Bulletin, **22**, 143 (1958).
4) H. Katagiri and T. Tochikura, This Bulletin, **23**, 482 (1959).
5) D. Cavallini, N. Frontali and G. Toschi, *Nature*, **163**, 568 (1949).
6) A. R. Jones, E. J. Dowling and W. J. Skraba, *Anal. Ghem.*, **25**, 394 (1953).
7) V. P. Calkins, *Anal. Chem.*, **15**, 762 (1943).
8) O. Warburg and W. Christian, *Biochem Z.*, **298**, 150 (1938).

bacterial glycollic acid oxidase was observed to be strongly inhibited by cyanide. It appears that this oxidase may be a metalloflavoprotein. The properties of the enzyme are now under investigation. Thus, it is suggested by the authors that the glyoxylate $\rightleftarrows$ glycollate system may function as a hydrogen carrier system in the respiration of microorganisms.

The authors wish to express their gratitude to Dr. H. Chiba and Mr. E. Sugimoto of Kyoto University for many helpful discussions and conscientious assistance.

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Received September 21, 1959

TABLE I

REACTIVATION OF GLYCOLLIC ACID OXIDASE APOENZYME OF *E. Coli* BY FLAVIN NUCLEOTIDES

Flavin nucleotides added (M/l)	Glyoxylate formed (μM/l)
None	0.35
Riboflavin-5'-phosphate, 1×10^{-4}	2.45
Riboflavin adenine dinucleotide, 5×10^{-5}	2.36

Reaction mixtures contained 600 μM phosphate buffer, pH 7.0, 500 μM semicarbazide, 500 μM Na-glycollate, 4 ml apoenzyme (prepared from 150 mg cells of strain G–2 of *E. coli* grown aerobically at 30°C for 20 hours on 1.5% Na-acetate plus 1% bouillon) and the requisite amounts of flavin nucleotides; total volume, 10 ml; 3 hours' incubation at 30°C on a shaker.

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162	left	37	10^{-4}	1×10^{-4}
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163	left	22	10^{-4}	1×10^{-4}
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383	left	foot note, 2	G-6-P glucose–6–phosphate	G-6-P, glucose-6-phosphate
384	right	23	-G1-P	G-1-P
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Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
(in Japanese)
Vol. 33, No. 10 (1959)

Studies on the Carbohydrates of the Seed of *Vicia faba*. Part V. Fucose as a Constituent of Hemicellulose B_1 from Broad-Bean Seeds. (p. *817~821*)

By Sin'itirô KAWAMURA and Teiiti NARASAKI
(*Department of Agricultural Chemistry, Faculty of Agriculture, Kagawa University*)

This is the detailed report corresponding to a preliminary report published in English (*Bull. Agr. Chem. Soc. Japan*, **22**, 436 (1958)). Furthermore fucose was identified by synthesizing crystalline 2,4-dinitrophenylhydroazone dioxane solvate by the solvent diffusion method of White and Secor (*Anal. Chem.*, **27**, 1016 (1955)). It is for the first time that fucose has been found as a constituent of any polysaccharide of leguminous seeds.

Secretion of Nucleotides by the Yeast Cell. Part II. On the Methods of Isolation and Identification of the Secreted Materials. (p. *821~826*)

By Masataka HIGUCHI and Teijirô UEMURA
(*Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University*)

It was previously reported that when yeast cells were incubated in a citrate buffer, nucleotides were released into the buffer from the cells. The methods of isolation and identification of the nucleotides secreted by the yeast was further investigated in this paper.

The secreted nucleotides were almost completely adsorbed by charcoal pre-treated with 8% isooctanol, and more than 80% of them was eluted with 0.3% NH_3–50% ethanol solution. The eluate from charcoal was neutralized with Dowex 50 (H^+ type) resin, and then passed through either Dowex 1 (formate type) or Dowex 2 (chloride type) resin column. The adsorbed nucleotides were separated with either formic acid and NH_4–formate (Dowex 1) or NaCl solution (Dowex 2) according to Hurlbert's gradient elution method.

While in the case of Dowex 1 resin column only about 60% of the total nucleotides adsorbed was recovered, the use of Dowex 2 resin column gave a good recovery rate (97%), although resulting in an indistinct resolution as to each of four mono-nucleotides and oligonucleotides.

Thus, Dowex 2 resin chromatography would be more useful to fractionate the mixture of mono-, oligo-, and poly-nucleotides such as these secreted materials.

Secretion of Nucleotides by the Yeast Cell. Part III. Fractionation of the Secreted Nucleotides by Ion Exchange Resin Column Chromatography. (p. *826~831*)

By Masataka HIGUCHI and Teijirô UEMURA
(*Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University*)

The nucleotides secreted by the P^{32}-labeled yeast cells were chromatographically fractionated by use of charcoal column and Dowex 2 (chloride type) resin column.

Seven fractions were obtained on the resulting chromatogram, where peaks of optical density were well agreeable with those of radioactivity, but not necessarily in respect of height. Mono-phosphates of adenosine, guanosine, uridine and cytidine, respectively, were identified in initial fractions (the eluates below 0.3M NaCl), then oligo-nucleotide fractions (0.3~0.5M NaCl) followed these mono-nucleotides, and finally the last fraction assumed as polynucleotides was eluted by mixture of 4.0M NaCl and 1M NaCl solution.

The base composition of the whole nucleotides secreted was as follows: adenine 1.0, guanine 0.82, uracil 0.72, cytosine 0.80. The ratio of purine bases to pyrimidine ones in the mono-nucleotide fraction was 2.5.

It is noteworthy that the poly- and oligo-nucleotide fractions occupied about more than 60 per cent of the whole nucleotides.

Amino acids and other ultra-violet absorbing materials than nucleotides were only a little contained in the secreted materials.

Studies on DL-Forming Lactic Acid Bacteria. Part II*. *Lactobacillus saké*, its Growth Factors and Racemiase Formation. (p. *832~835*)

By Akira ÔBAYASHI and Kakuo KITAHARA
(*Institute of Applied Microbiology, University of Tokyo*)

All strains of *Lactobacillus saké* were unable to grow in a semisynthetic medium which is generally used for lactic acid bacteria. Presence of some factor(s) essential for its growth was experimentally suggested in yeast extract, peptone, koji-extract and saké. Enzymatic digests of casein by crystalline trypsin or by protease of

Aspergillus oryzae were also active indicating that the active substance(s) is a peptidic nature.

From the tryptic hydrolysate of casein, two active factors were fractionated using paper chromatography together with bioautography. By hyprolyzing with 6N HCl at 100 for 12 hours, we found the factors were phenylalanyl peptides the one was consisted from eight amino acids, namely, phenylalanine, leucine (or isoleucine), valine, proline, alanine, threonine, glycine and lysine, and the another contained methionine in addition to these eight amino abids.

Several active peptides were detected in saké and polypeptone (fungal digest of casein), however, they were different from those in the tryptic digest.

Peptidic factor which is essential for growth of *L. saké* is therefore not limited to a single definite substance.

L. saké reveals a good growth in the semisynthetic medium supplemented by a peptidic factor, but acetate must be eliminated from this medium for synthesis of racemiase.

* Studies on the Enzymes of Lactic Acid Bacteria. Part XV.

Studies on DL-Forming Lactic Acid Bacteria. Part III*. Formation of Apo-racemiase in *Lactobacillus saké*. (p. *835~839*)

By Akira ÔBAYASHI and Kakuo KITAHARA
(*Institute of Applied Microbiology, University of Tokyo*)

We investigated the formation of racemiase using *Lactobacillus saké*, which produces various optical forms of lactic acid (L-, L+DL- and DL) depending upon cultural conditions, such as the type of nitrogen or carbon sources. The formation of this enzyme was inhibited by the addition of Na-acetate.

Interesting was the effect of lactate. The formation of racemiase was stimulated by the addition of L-lactate which is naturally produced by this species, while the process was markedly suppressed by the addition of D- or DL-lactate to the medium. It should be noted that the synthesis of racemiase is inversely affected by L- and D- lactate, whereas the enzyme once synthesized it can act on both of D- and L-enantiomorphs with almost equal activity.

When arabinose was used as a sole source of carbon, *L. saké*, could never produce racemiase, regardless of the nitrogen sources. This may be because racemiase formation was inhibited by the acetate produced with lactate in equimolar quantities from arabinose. When L-lactate was added to the media containing arabinose, the suppressive effect of acetate was overcome by L-lactate, leading to the formation of racemiase.

In the case of *L. saké* L-lactate acts as an essential factor invoking the formation of racemiase, while D- or DL-acid has an inhibitory effect upon the process of enzyme formation. From this fact we suggested that racemiase in *L. saké* is an enzyme which protects the bacterial cells by mitigating the possively injurious action of its metabolic product, L-lactate.

* Studies on the Enzymes of Lactic Acid Bacteria. Part XVI.

On the Factor 'Determining the Flora of Lactic Acid Bacteria in the Starter of Saké. (p. *839~843*)

By Akira ÔBAYASHI and Kakuo KITAHARA
(*Institute of Applied Microbiology, University of Tokyo*)

It is very interesting from oecological point of view, that the flora of lactic acid bacteria naturally occurring in the preparation of 'moto', the starter of saké, is restricted to only two species, namely *Leuconostoc mesenteroides* and *Lactobacillus saké*,

In this paper, result of experiments on the reason of this phenomenon is presented.

Effective peptides, originating from koji-digestion, for growth of *L. saké* appears to be richly present in the moto, however, this is insufficient as the reason why the other species can be scarcely found in it.

It was found that the two species can grow under comparatively low temperature, viz. 4°C, which is incapable for other species.

Preparation of moto is usually carried out at low temperature such as 5~10°C, therefore, ihe cause of the predomination of these two species is concluded to be nothing but their growth temperature.

An occasional contamination of *L. batatas* and certain species belonging to *Pediococcus* and *Streptococcus* may also be explained from the fact that these species come after the *Leuconostoc* and *L. saké* in their capabilities to grow at lower temperatures.

Formation of L-Glutamic Acid from Fumaric Acid by Bacteria. Part I. On Isolation and Identification of Suitable Bacterial Strains. (p. *843~845*)

By Ryohei AOKI, Yasuhiro KONDO, Toshinao TSUNODA and Tetsuo OGAWA
(*The Central Research Laboratory of Ajinomoto Co., Inc.*)

(1) Many bacteria which produce L-glutamic acid from fumaric acid were isolated from the natural sources. The best strain among them, F_{6-1} 159, has accumulated 2.94 g/dl (74% based on the initial fumaric acid) in fermentation broth by shaking culture at 30°C for 72 hrs., in the following medium.

Fumaric acid	4 g/dl
Glucose	3
KH_2PO_4	0.1
$MgSO_4 \cdot 7H_2O$	0.02
Protein hydrolysate	0.008 (as nitrogen)

Neutralized with NH_3 and KOH*

(2) The strain, F_{6-1} 139, was identified with *Bacillus pumilus*.

* One mole of potassium hydroxide to 1 mole of fumaric acid is added and the resulting medium is adjusted to pH 7 with ammonia.

Studies on the Proteins of Insect Hemolymph. Part V. Association-Dissociation (2). (p. *845~849*)

By Junko ODA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

In order to elucidate the association-dissociation of body fluid proteins of Bombx mori, the sedimentation of these proteins was studied over a wide pH range from 3 to 10, and at different ionic strengths, 0.03~1.0. In addition, the molecular weights of these proteins were determined by light scattering method in various conditions.

The results obtained provided an additional evidence concerning the association-dissociation of these proteins; e.g., a component having S (sedimentation constant in Svedberg's unit) of 15 dissociated into two components having S of 7 and 5 respectively, and a component having 7 S converted to 5 S and 2.5 S components. The dissociation of these proteins was confirmed to occur depending upon their characteristic properties and not due to the incidental degradation these proteins during their preparation. Metal ions such as Ca^{++}, Mg^{++}, and $Fe(CN)^{++++}$ did scarcely effect on the dissociation of these proteins. In a concentrated urea solution, a remarkable increase in particle weight was observed in acid and neural pH ranges. However, in an alkaline pH range proteins were rather slightly dissociated. Organic reagents such as glycerin and glucose did not affect the intensity of light scattered by these proteins.

Studies on the Proteins of Insect Hemolymph. Part VI. Salting-out Behavior. (p. *850~853*)

By Junko ODA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

An attempt was made of the preparation of body fluid proteins of Bombyx mori by salting out with ammonium-, sodium-, magnesium sulfate, potassium phosphate, and sodium chloride respectively.

A bulk of proteins was precipitated on the addition of ammonium-, sodium sulfate, and potassium phosphate respectively to body fluid protein solution. The same shaped ionic strength as solubility curve was obtained with any of these salts. Two knicks were observed on the curve, one was a considerably deffnite, while another was at pH higher than 5 and indistict. However, in cases of magnesium sulfate and sodium chloride, only about 20 per cent of the proteins were precipitated even at a saturated concentration of the salt in neutral pH range, whereas in acid pH range, large part of the proteins was precipitated.

Any fraction obtained by salting out with each salt was not homogeneous, but a mixture of three or more protein components.

From these results, the salting out procedure with neutral salt seemed to be unfavorable to the preparation of body fluid protein of the silk worm. The difficulty in purification of these proteins might occur due to the coprecipitation resulted from the association-dissociation and intermolecular interaction of the proteins.

At a constant ionic strength, the pH-solubility curve had a maximum and a minimum point remarkably at low pH's. This seemed likely to indicate a certain change in protein configuration.

Studies on the Proteins of Insect Hemolymph. Part VII. Heat Denaturation. (p. *853~857*)

By Junko ODA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

In order to characterize the body fluid proteins of Bombyx mori, the heat stability of these proteins was examined.

The effects of temperature, pH, and protein concentration on the coagulation of the proteins by heating in various buffer solution were studied. At pH 3, coagulation occurred rapidly forming gel. At 70°C, about 80 per cent of the proteins of body fluid became insoluble at pH 3, while about 60 per cent at pH 4~7. There was apparently no change in solubility of proteins at 70°C to 80°C. However, at pH 9, coagulation did not occur in any temperature between 50° and 100°C. Amount of proteins remained in solution by heating were decreased depending upon the increase in protein concentration used. Both protein solutions heated either at 70°C or 80°C for 5 min. were analyzed by ultracentrifuge and electrophoresis. It was revealed in each temperature that the protein components either

of the largest or the smallest sedimentation rate remained in solution without accompanying the change in their molecular weight as well as net charges, while the middle component disappeared from the solution. The protein, which did not coagulate by heating at 100°C and was separated by the precipitation with ethanol, was revealed to show a single sedimentation boundary in phosphate buffer solution of pH 7 and ionic strength 0.1. Its sedimentation constant was approximately 0.95×10^{-13}. However, this protein was not confirmed to be homogeneous by electrophoresis.

Studies on the Constitution of "Rice-koji" Protease. Part I. Investigation by Adsorption and Zone Electrophoresis of Enzymes. (p. *857~861*)

By Meiji SUZUKI, Yataro NUNOKAWA and Yutaro ITO

(*Brewing Experiment Station, Tax Administration Agency*)

For the purpose of examining the constitution of protease of "rice-koji", aqueous extract of "rice-koji", which was incubated avoid of contamination, was prepared. After partial purification of enzymes in the extract by sedimentation with conc. alcohol, the precipitation was re-dissolved into buffer solution.

The purified and concentrated enzyme solution was used for the above mentioned purpose.

1) As the amount of acid protease was very large, it was difficult to demonstrate distinctly the other proteases in "rice-koji", only by electrophoresis.

2) Among inhibitors and adsorbents so far examined, alumina-gel seemed to be the best to eliminate the acid protease from the enzyme solution.

3) By electrophoresis we found the enzyme solution, which lacked the acid protease by treatment with alumina-gel, contained three types of proteases.

4) We came to the conclusion ihat at least four types of proteases were contained in "rice-koji"; the first was the acid protease, the second was the alkaline protease and the last two were the enzymes which were active in wide ranges of pH, around neutrality. And one of whidh was the newly recognized enzyme.

Microdiffusion Analysis for Determination of Total Nitrogen in Shoyu. Studies on Application to the Analysis of Shoyu and on the Kjeldahl's Digestion Procedure as adopted by the Shoyu Technical Society of Japan. (p. *862~866*)

By Yuzuru KUME, Yoichi IMURA, Sadatoshi YAMASHITA and Toshitake SHIMATSU

(*Fundokin Shoyu Co., Ltd.*)

A study has been made on the reliability, precision and accuracy about the microdiffusion analysis developed by Conwey for determing the total nitrogen in shoyu. Statistical evaluation showed that the assays can be repeated with good reproducibility.

These results were comparable to those obtained by the Kjeldahl's method which had been adopted by the Shoyu Technical Society of Japan, and rather the latter yields values possessing lower averages and higher errors. From the analysis of the errors, it was demonstrated that the assignable cause depends upon some unfit prescriptions of the significant factors which are involved in the Kjeldahl's digestion procedure.

The suitable procedure, to be used only for shoyu, was a modification of the A O A C method. This gave a coefficient variation of $\pm 0.057\%$.

Studies on Reticulo-rumen Digestion. Part XIV. On the Digestion of Starch in the Rumen of Sheep. (p. *867~871*)

By Fumio KUMENO and Makoto KANDATSU

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

There are three ways by which starch is broken down by the ruminant; some will pass directly to the abomasum without being attacked by rumen microbes and undergo normal digestion as in other animals; some will be ingested by the microorganismus and preserved as polysaccharides which may be conveyed to the host in one form or another; while some will be subjected to fermentation and converted to volatile fatty acids, these then being absorbed by the host. It is the purpose of the present study to determine the proportion of starch accounted for by each of these three routes, and the rate of its disappearance in the rumen.

Two experimental diets were fed to sheep. The first, diet A, consisted of: 100 g hay and 200 g starch; while the second, diet B, consisted of: 100 g hay, 200 g starch and 120 g soy bean oil meal.

The rumen contents, withdrawn through a permanent rumen fistula before and at appropriate intervals after feeding, were divided into several fractions by centrifugation. The proportion of starch accounted for, by microbial convertion into polysaccharides and soluble sugars, increased as digestion of starch proceeded, while the proportion of undigested starch rapidly decreased.

Polysaccharides, produced by bacteria and protozoa, increased from 0.03~0.13 g/100 g rumen content to 0.11~0.44 g/100 g; and soluble sugar increased from 0.001 g to 0.03~0.11 g/100 g rumen content, in 8 hours

after feeding.

Recovery of starch from the rumen at 1 hour after feeding was 42～64% on diet A and 29～39% on diet B; these values were calculated on the assumption that total rumen content is 8 kg. However, recovery of starch by withdrawing the entire rumen content through the rumen fistula after 1 hour and 8 hours was 58～77% and 5～18% respectively. These results suggest that a greater part of starch ingested with hay passes directly to the abomasum without undergoing microbial fermentation.

Studies on Changes in Stored Shell Eggs. Part II. Fractionation of the Egg White by Chemical Method and Changes of its Constituent during Storage. (p. *872～875*)

By Yasushi SATO and Ryo NAKAMURA

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Nagoya*)

One method was devised to separate a chicken egg white into proteins and non-protein nitrogen compounds by the chemical methods. This method was applied to the analysis of the stored shell eggs. The results of its analysis revealed that the protein of the chicken eggs were not decomposed into small fractions, but they changed among them during storage; that is, the amount of ovomucin and ouomucoid decreased, and the amount of other proteins except these increased gradually.

Thinking from these results together with our previous report on the electrophoretic analysis of the stored shell eggs, it is inferred that thinning changes of the dense white involve the changes of conalbumin into globulin-like protein.

Studies on Esterases from Seeds. Part IV. Activation of Esterase from Rice-embryo. (p. *876～880*)

By Tetsujiro OBARA and Yasokichi OGASAWARA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University of Education*)

It has been shown in a previous report that the esterase from rice-embryo is inactivated, after dialysis and electrophoresis. Consequently, we could not estimate the activity of the esterase. Consequently, we could not estimate the activity of the esterase.

Now, we have carried out experiments on the activation of the esterase in order to estimate the activity without this inconvenience.

In these experiments, we have examined the activation of the crude solution of the dialyzed esterase by the method of the ultraviolet absorption spectra.

The results of experiments mentioned above were as follows:

(1) The pH optimum of the enzymatic reacion of the embryoesterase by the *p*-nitrophenol method was about 7.38.

(2) We found that several divalent cations had activating effects on this esterase. The potency of the activation increased in the order——$Mg^{++}<Ba^{++}<Mn^{++}<Sr^{++}<Ca^{++}$.

The activation of the esterase by Co^{++} or Ni^{++} was very weak.

(3) Activating substances were contained in the crude solution from rice-embryo.

(4) We found that *p*-nitrophenyl acetic acid was decomposed not only by the esterase itself but also by substances in the crude solution.

Studies on Myrosinase. Part III. Distribution of Myrosinase in Plants and Activity-ratios of Myrosulfatase and Thioglucosidase exhibited by Myrosinase obtained from Different Origins. (p. *881～885*)

By Zenji NAGASHIMA and Masaaki UCHIYAMA

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Shizuoka*)

Myrosinase has been recognized by Neuberg to be a mixture of myrosulfatase and thioglucosidase which act individually on mustard oil glucoside. If that is true, the activity-ratios of myrosulfatase and thioglucosidase exhibited by myrosinase obtained from different origins must not be constant. The present work was undertaken to examine this point.

First, a method to determine the activity of myrosinase was set up in which, allylisothiocyanate produced in the reaction of this enzyme on sinigrin was derived to allylthiourea and the optical absorption ($E_{237m\mu}$) of the aqueous solution of allylthiourea indicated the activity of this enzyme.

Secondary, the enzyme was found to be much activated by the addition of ascorbic acid, which was used on the determination of enzyme activity when needed.

The distribution of myrosinase in plants was examined by the method described above and it was found that myrosinase was contained in all samples of Cruciferae and presented in leaves, stems, roots and all other portions of these plants.

The activity-ratios of myrosulfatase and thioglucosidase were all approximately one in samples containing myrosinase.

Studies on the Fermented Milk. Part I. Studies on Stabilizers of the Sour Milk Drink containing

Natural Fruit Juice (I). Effect of the Esterification Degree of Propylene Glycol Alginate. (p. *885~889*)

By Toyozo OOKI

(*Research Institute of Calpis Food Industry Co., Ltd.*)

Propylene glycol alginate (I) has an excellent property as a stabilizer of the fermented sour milk drink containing natural fruit juice (II). But it is found that its stabilizing effect varied from lot to lot. To elucidate this problem, 15 samples of I on the market were analysed for sodium alginate, fraction (III), alginic acid fraction (IV), and alginic acid ester fraction (V). The results obtained that III was 3.25~5.57%, IV 2.32~7.20% and V 75.2~83.2%. II, which was prepared from each lot of I, was kept in an incubator at 37°C to examine its stability. A comparison between the stability of II and the amounts of III, IV and V in propylen glycol alginate samples indicated that the larger the amount of V, the higher the stability of II. It was concluded that the stability of II depends on the esterification degree of I.

The Utilization of 1,2-Propylene Glycol by Microorganisms. (p. *889~893*)

By Makoto ISHII, Tokuya HARADA and Ziro NIKUNI

(*Institute of Scientific and Industrial Research, Osaka University*)

Attempts to have a proteinous animal fodder or to have some intermediate products from 1,2-propylene glycol by microorganisms were made.

A number of microorganisms, 2 strains of yeast, 6 strains of bacterium and 12 strains of mold, were isolated from soil. All of them could propagate in a medium containg 1,2-propylene glycol as a sole carbon source.

Using a strain of the yeast (Y_1), culture conditions to have the best yield of the yeast were examined. When Y_1 was cultivated in a medium containing 1% of 1,2-propylene glycol as a sole carbon source and 0.5% of ammonium salt at 30°C for 48 hours under shaking, over 90% of the glycol was consumed and about 30% of its weight was recovered as dry yeast powder.

A part of 1,2-propylene glycol was converted to lactic acid and to some unknown organic acids by the the yeast.

Studies on the Nutritive Value of Grass Proteins. Part VIII. The Effect of Drying Methods upon the Digestibility of Soy Bean Leaf Proteins. (3) The Effect of Drying Method with Aeration at 40~50°. (p. *893~898*)

By Makoto KANDATSU and Tadahiko YASUI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

The changes in some conststuents of Soy bean leaf dried with aeration at low temperature were followed. The loss of crude protein during drying was small and those of the others were almost none. The digestibilities of protein and Nitrogen free Extracts were lowered about 2 and 5 per cent respectively by drying, but digestibility coefficient of crude fiber was higher than that of the fresh leaf and those of the others were about 3~5 per cent lower than those of the fresh one. According to drying, water soluble, 10% NaCl soluble and 0.3% NaOH soluble proteins decreased remarkably and insoluble proteins increased very much. At the same time, the apparent digestibilities of the former three proteins were lowered and that of the latter was risen. From the results of our experiment, it was suggested that the decrease of apparent digestibility of protein may be caused by considerable increase of metabolic fecal nitrogen excretion in the feces.

Studies on the Nutritive Value of Grass Proteins. Part VIII. The Effect of Drying Methods upon the Digestibility of Soy Bean Leaf Proteins. (4) The Effect of Drying in the Sun. (p. *899~903*)

By Makoto KANDATSU and Tadahiko YASUI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

The changes in some constituent of Soy bean leaf dried in the sun were followed. The Nitrogen free extracts were much, but the others were slight lost. Neverthless, the distribution of proteins in the leaf underwent remarkable changes, that is, 0.3% alkaline soluble protein increased remarkably, whereas the water soluble, 10% saline soluble and insoluble proteins decreased.

Although apparent digestibility coefficient of crude fiber was even higher than that of fresh one, those of protein and Nitrogen-free extracts were lowered as compaired with those of fresh leaf and dried leaf with aeration at low temperature, especially, those of 0.3% alkaline soluble, water soluble and insoluble proteins were lowered, whereas that of 0.3% alkaline 60% hot alcohol soluble protein did not change.

It was suggested from the results obtained that the decrease apparent digestibilities of proteins may be caused not only by the changes of native proteins into undigestible ones owing to reaction with carbohydrate during drying, but also by the considerable increase of metabolic fecal nitrogen excretion in the feces.

Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
(in Japanese)
Vol. 33, No. 11 (1959)

Studies on the Aspergilli for Saké Brewing. Part VII. On the Identification of the Aspergilli for Saké Brewing and their Oxygen Adaptation. (p. *905~909*)

By Hideya MURAKAMI and Kiyomi TAKAGI

(*Brewing Experiment Station*)

The *Asp. oryzae* var. *saké* named tentatively by the authers was identified on the *Asp. oryzae* var. *globosus* by Sakaguchi et Yamada's classification on the several grounds as follows: the morphological characteristics, the great oxygen demands and the production of chestnut like aroma.

The lack of oxygen induenced both on the morphological and physiological characters of molds and on the action of diastase, for instances, the aerobic types of molds showed the properties of the anaerobic types and the diastase action decreased its activities.

The authors explained the reason why the wet koji made by the little supply of the air had always the higher acidities than the dry koji made by the large supply of the air, and considered the several other phenomena in saké qrewing on these various grounds.

Studies on the Fermented Milk. Part II. Studies on Stabilizers of the Sour Milk Drink containing Natural Fruit Juice (2). The Effect of Sodium Alginate and Alginic Acid Fraction on the Stability of this Drink, and the Process for the Improvement of the Stabilizing Action of P.G.A. by adding Mg-salts. (p. *909~913*)

By Toyozo OOKI

(*Research Institute of Calpis Food Industry Co., Ltd.*)

Propylene glycol alginate (I) which had been esterificated at relatively high degree was mixed with alginic acid (II) at the rate of 1~5%, and also I was mixed with sodium alginate (III) at the same rate. Using such stabilizers, the sour milk drinks containing natural fruit juice (IV) were prepared and the effect of II and III on the stability of IV was examined. As the result, the addition of II to I or III to I accelerated the settling of IV and the addition of III to I formed more coagulation than that of II to I from IV.

It was found that Mg-salt prevents the coagulation from this drink due to alginic acid fraction and sodium alginate fraction which are fractions of non-esterification of I. At the extent of 0.05~0.20% as $MgSO_4 \cdot 7H_2O$ to IV, the addition of 0.2% $MgSO_4 \cdot 7H_2O$ is most useful in improving the stability of IV, and makes the stability of IV prepared with I of low degree of esterification nearly the same as stability of IV prepared with I of high degree of esterification.

On the Tannins in Green Tea. Tea Tannin II (Continued). (p. *914~915*)

By Michiyo TSUJIMURA and Naoko OSAWA

(*Laboratory of Food Chemistry, Jissen Women's College*)

The author continuing research in order to ascertain the melting point and analytical result of the tea tannin II or *l*-epigallocatechingallate $C_{22}H_{18}O_{11}$, has become convinced that, although the melting point of tea tannin II differs due to its different conditions, its analytical result agrees with the calculated value of $C_{22}H_{18}O_{11}+H_2O$.

As to the acetyl derivative of tea tannin II, its analytical result agrees with $C_{22}H_{10}O_3\ (OCOCH_3)_8+H_2O$.

From this result, it is understood that the tea tannin II isolated by the author from Japanese green tea, contains one molecule of water.

Studies on Pungent Principles of Red Pepper. Part IV. Paper Chromatography of the Pungent Principles. (p. *915~918*)

By Sadayoshi KOSUGE, Yukio INAGAKI and Michio NISHIMURA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Gifu University*)

Studies on paper chromatography of capsaicin, a mixture of two pungent principles, and of the two were carried out, because these studies had not been carried out up to this time.

I Paper.
Toyo filter paper No. 50.

II Developement.
Uni-dimensional ascending method.

III Developers.
For capsaicin use. Benzene: methanol=30:1.

For two principles use. Many developers were found. Benzene: butanol: water=9:1:10 (underlayer liquor was used). Benzene: ethanol: water=4:1:1 (underlayer liquor was used). 0.2M-NaOH: 0.1M-Na_2CO_4: 0.4M-$NaC_2H_3O_2$=3:2:5. 0.1M-NaOH. 0.1M-NH_4OH: 0.05M-Na_2CO_3=1:1. acetic acid: water=3:17. 0.1M-NaOH: 0.05M-Na_2CO_3=3:2.

Color reagents. The following two were used.

Folin-Ciocalteu's reagent: After phosphomolybdic-tungstate solution was sprayed, blue spots by the pungent principles were developed by means of spraying 1.5M-Na_2CO_3.

Ferric Chloride-Potassium Ferricyanide reaction: After a mixture of 1% ferric chloride and 1% potassium ferricyanide (1 : 1) was sprayed, blue spots by the principles were developed by means of spraying 0.5N-HCl.

Studies on Pungent Principles of Red Pepper. Part V. Determination of the Pungent Principles (2). Ultraviolet Spectrophotometric Method. (p. *918~921*)

By Sadayoshi KOSUGE and Yukio INAGAKI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Gifu University*)

As simpler and more accurate methods for the quantitative determination of the pungent principles, was tested the direct procedure based on the ultraviolet absorption of these compounds.

Capsaicin, a mixture of two pungent principles, is extracted with ethylether, and, after evaporation of solvent, is taken up in carbon tetrachloaide. The capsaicin in carbon tetrachloride, after being washed with dil-acetic acid, is taken up in 0.5N-sodium hydroxide. The absorbancy of the obtained capsaicin solutioh is measured at the wave-length of 246 mμ or 295 mμ.

No difference is found in pungency and ultraviolet absorbancy of pungent principles I and II. Accordingly, capsaicin, a mixture of principles I and II, has only to be analysed for acrid condiment.

Electro-desalting from the Protein Degradated Products in Foodstuffs by using Ion Permeable Membranes. (p. *922~926*)

By Akira KAWAMURA and Kiso AKABANE

(*Department of Agricultural Chemistry, Tokyo Noko Daigaku*)

When the analyses of the food compornents are intended, the existence of high concentrated NaCl occasionally interfers the results of them, for example, analyses.

In this report, through the electrodialysis, the authors compare the desalting efficiencies of two kinds of ion permeable membranes (homo- and hetero-types) with that of cerophane membrane by using amino acid-NaCl mixtures and Miso (a salted foodstuff having degradated soybean protein) as the samples.

For the experiments, the authors recognize that the both ion permeable membranes (I.P.M.) are superior on the efficiencies of desalting to the cerophane. In I.P.M., the homo-type one can take 6.0~7.0 hrs until to lose 80% of NaCl in the samples but the loss of nitrogen is rather low. On the contrary, the hetero-type one (Amberplex A-1, C-1) can lose 97~99% of NaCl within 1.5~2.0 hrs but the loss of nitrogen is rather high.

When the pH of samples are held on slight alkali side and the desalting degrees are discontinued within 90~95%, during the electrodialysis by Amberplexes, the loss of nitrogen from Miso can be made 10% or so. And they can decide that the hetero-type I.P.M. may be useful in the case without taking aim at the perfect desalting.

Formation of L-Glutamic Acid from Fumaric Acid by Bacteria. Part II. On Stabilizing and Increasing of the Yield by Heat Shock. (p. *927~931*)

By Ryohei AOKI, Yasuhiro KONDO, Toshinao TSUNODA and Tetsuo OGAWA

(*The Central Research Laboratory of Ajinomoto Co., Inc.*)

(1) The ability of the best strain, F_{6-1} 139, to accumulate L-glutamic acid, was very unstable, but it has been stabilized by a heat shock of the cell suspension for 10 min. at 60°, and by plating it on the following medium:

Fumaric acid	1 g/dl
Glucose	0.5
Meat extract	1
Peptone	1
Agar	2

Neutralized with NaOH to pH 7.

(2) The amount of L-glutamic acid, which is accumulated in fermentation broth, has been increased by a heat shock for 20 min. at 60° immediately after inoculation.

(3) It was ascertained that the effect of these heat shocks were not to weed out vegetative cells.

The Change of Properties of Potatostarch Granule by γ-Irradiation. (p. *931~936*)

By Akira MISHINA and Ziro NIKUNI

(*The Institute of Scientific and Industrial Research, Osaka University*)

Air dried Potatostarch granule containing 18.5% moisture were irradiated from a source of 1,400 C^{60} Co with different dose of 10^5~10^8 γ.

By the irradiation, intrinsic viscosity, viscoelasticity measured by V.I. viscometer, and blue value of the starch decreased.

Reducing power, alkali L-ability number, carboxyl value, carbonyl number and susceptibility by β-amylase of the specimen increased corresponding to radiation doses.

In the irradiated starch specimen, glucose, maltose and a series of small dextrin were recognized by paper chromatographic analysis.

These results indicated that hydrolysis and oxydation of the starch specimen occured during the radiation. Therefore, Potatostarch was considered as a γ-ray degradation type polymer as well as cellulose.

X-ray diffraction diagrams showed that crystalline part of the Potatostarch was damaged with the dose up to $1.5\times10^7\,\gamma$:

All those change in properties were lowered by the irradiation under a diminished pressure.

Antibiotic Production by Spore-forming Bacteria. Part V. On the 'Protoplast Dissolving Factor' (1). (p. *936~940*)

By Tokujiro AIDA and Kunio KATO

(*Laboratory of Agricultural Microbiology, Faculty of Agriculture, Tohoku University, Sendai, Japan*)

The culture filtrate of *B. subtilis* st. 427~4 showed marked lytic activities against not only the intact cells of the bacitracin producer (*B. subtilis* st. Tracy) but also the protoplasts of it. The substance responsible for the latter activity, propositionally named by the authors 'protoplast dissolving factor' (PD factor), was not dialyzable but precipitable either with 0.6 saturated ammonium sulfate or under the acidic condition (pH 4.2).

The PD factor seems to be an enzyme-like substance, but its partially purified preparation exhibited no significant activities of several hydrolytic enzymes such as protease, lipase, ribonuclease known to dissolve the bacterial protoplasts. Further, the factor was noe inactivated by heating (100°, 10 min.), whereas the lytic activity against the intact or heat-killed cells were thoroughly lost by this treatment. The factor, then, differs in its nature from the lytic enzyme against intact cells.

The mode of action of this factor is as yet obscure but its physiological significance is well emphasized because of the fact that the PD factor-treated protoplasts of *B. subtilis* st. Tracy no longer produced any of the bacitracin-active substances, although its intact protoplasts could do so as recognized previously.

Studies on Light Scattering by Cell Suspensions. (1) Total Scattering Cross Sections for Spherical Cells. (p. *941~946*)

By Shozo KOGA and Teruyuki FUJITA

(*Institute of Applied Microbiology, University of Tokyo*)

Three methods are proposed for the evaluation of the cellular refractive index. Unicellular organisms in aqueous suspensions are found to make "soft scatterers" with the relative indices of refraction 1.04 to 1.05. The total scattering cross section of a single spherical cell is, therefore, expressed by Hart-Montroll formula as a function of its relative index of refraction, averaged cell diameter and the wavelength of incident light. The observed values for *Saccharomyces cerevisiae*, *Rhodotorula glutinis* and *Micrococcus sphaeroides* are shown to fit fairly well the theoretical curve. Some remarks are thereby given with regard to the microbiological application of the microbiological application of turbidimetry.

Distribution of Riboflavin in the Kernel of Rice during the Ripening Period of Rice Plant. (p. *946~949*)

By Kiyohide SONE

(*Department of Living Science, Faculty of Agriculture, Tohoku University*)

The fluorometric method of riboflavin determination was applied for histochemical investigation, and the riboflavin localization in tissues of rice kernel was observed in connection with the development of kernel after flowering.

The development of embryo and endosperm were completed almost by the early part of ripening period. Riboflavin distribution in rice kernel seemed to be set within one week in parallel with the development of a kernel, and then, intensity of riboflavin fluorescene became higher especially in pericarp tissue and aleurone layer. The fluorescene became more luminous as the ripening progressed, and was finally set the intensity around the 30th day after flowering.

Riboflavin in endosperm at this time was homogeneously located in each tissues except the part near embryo which gave the high intensity of riboflavin fluorescence. As to the riboflavin localization in embryo, great deal of riboflavin was observed in vascular-bundle of mesocotyl and that of primary-root, and plumular leaf which becomes to the main part of rice plant in future.

Studies on the Film-soja-yeast. Part IV. Influence of Surface Active Agents on Film Formation. (p. *949~956*)

By Toshio NAKAHAMA and Hirotsugu IMAHARA

(*Faculty of Agriculture, Kyoto Preffectural University*)

Zygosaccharomyces salsus grew with forming film on the basal medium supplemented by inorganic salt or sugar, but formed film neither on the basal medium itself nor on the medium containing sodium chloride with a surface active agent. Other film formers such as *Hansenula anomala*, *Pichia belgica* or *P. membranaefaciens* also did not form film on the media which were added surface active agents.

Thus the surface active agents changed the state of growth of film formers significantly.

We used Tween 20, 40, 60 and 80 as surface active, non ionic agents at the concentrations from 0.001 to 4.0 percent.

When salt or sugar was added, the surface tension of the medium was somewhat increased, whereas it was markedly decreased when the surface activator was added.

When some film-forming yeasts were cultured at the conditions which does not form film, the amount of alcohol produced in the media was three to five times as much as the amount produced at the conditions of forming fim.

Some film-forming yeasts did not form films the begining of culture on the media which had been added the surface active agents, however, became to form films in the course of prolonged incubation. This phenomenon would be caused by gradual decomposition of the surface activator by the action of yeast enzyme. Fatty acid moieties of the surface active substances were proved to have no effect on the state of growth of yeasts.

On the Development of Turbidity of Calcium-soluble Fraction of α-Casein by Rennin. (Studies on Milk Coagulating Enzymes. XII) (p. *957～961*)

By Kunio YAMAUCHI and Tomokichi TSUGO

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

Development of turbidity of calcium-soluble fraction of Warner's casein (κ-casein fraction) by rennin was studied by measuring transmission without no filter at various conditions. The fraction used was 0.4% solution of pH 6.6 and containing 0.085M NaCl. The transmission curve consisted of three phases; the initial phase in which no marked turbidity was developed, the second phase in which turbidity increased rapidly and the final phase in which optical density reached almost maximum. The logarithm of the difference between maxium value of optical density (D_∞) and each value (D_t) at t minutes after rennin addition was rectilinearly related with time (t) except for the initial phase; $\log\left(\frac{D_\infty - D_t}{D_\infty}\times 100\right) = -K(t-t_i)+2$ (K, t_i: constant). The measure of the slope, that is K, was proportionally increased with increment of added rennin concentration within certain limit of rennin concentration. Temperature coefficient (Q) for the reaction was calculated from K values at different temperatures (15～41°). The mean of $Q_{1°C}$ was about 1.08, which was the same extent as that of general enzyme reaction and not so high as that of protein denaturation. The change of calcium-soluble fraction was assumed to consist of two processes overlapped each others, that is enzymic transformation of κ-casein into para κ-casein and the aggregation process of the latter. As D_∞ was increased with temperature, the latter process itself was also considered to be dependent on temperature. Horse serum was found to inhibit the development of turbidity. The inhibition was greater when the serum was added before rennin addition than when added at the beginning of the second phase.

Studies on the Proteins of Insect Hemolymph. Part VIII. Electrophoretic Behavior in Acid Solutions. (p. *961～965*)

By Junko ODA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

Electrophoretic patterns as well as mobilities of body fluid proteins of Bombyx mori were examined by carrying out the electrophoresis using different concentations of proteins at different temperatures, in various buffer solutions of different pH's and ionic strengths.

The effect of pH and concentration of buffer solutions on the electrophoretic behavior of the proteins were on complexity. For instance, two peaks, i.e., a high, sharp, and rapidly moving peak and another low, slowly moving peak in the ascending pattern and only a diffused boundary in the descending pattern were observed in the electrophoresis carried out at 11 to 13°C in sodium phosphate-citric acid buffer solutions of pH 3 and ionic strength below 0.02 as well as above 0.2, while ionic strength between 0.03 and 0.2, there were three or more peaks. Hence, it was concluded that in this region of ionic strength, area of each peak would change depending upon ionic strength, composition and particularly concentration of buffer solution employed. Different electrophoretic patterns were also obtained in different buffer solutions. These evidences seem likely

to suggest that protein molecule will change its configuration as the results of a change in the interaction between protein molecule and the surrounding media and subsequent change in electrophoretic mobility. No exceptional effects of protein concentration and temperature on the electrophoretic pattern were observed. So-called boundary anomaly in electrophoresis at low ionic strength was scarcely observed in this experiment.

Studies on the Proteins of Insect Hemolymph. Part IX. Chromatography on Calcium Phosphate Columns. (p. *965~971*)

By Junko ODA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

For the purpose of the separation of each protein contained in body fluid of Bombyx mori, column chromatography with hydroxylapatite was carried out in various conditions.

The proteins in 0.005 to 0.02 M phosphate buffer solution mainly of pH 6.8 were applied to the column with diameter either of 10 or 15 mm. Stepwise elution was carried out with increasing concentration of phosphate buffer. Some of the experiments were performed in phosphate buffer solution of different pH's.

It was found that although the chromatography were performed in exactly the same manner with the adsorbent prepared by strictly the same procedure, the number and the area of the peaks obtained in the effluent were so variable that three or five peaks resulted. In other words, the chromatograms of the proteins contained in silk worm were not reproducible, whereas that of crystalline bovine serum albumin was confirmed to give a reproducible result.

From these results, it was concluded that such a instability of the body fluid proteins of silk worm will depend upon the characteristic properties of these proteins and not due to the variety of the experimental conditions.

Studies on the Proteins of Insect Hemolymph. Part X. Fractionation with Calcium Phosphate. (p. *971~977*)

By Junko ODA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

In order to isolate the several proteins from body fluid of Bombyx mori, the body fluid solution was chromatographed by passage through a column of hydroxylapatite. In addition, to examine the homogeneity of each component fractionated in the effluent, its sedimentation and electrophoretic patterns were observed in the usual manner. For this purpose, each fraction was concentrated up to 50 to 100 times concentration fly adsorption-elution procedure on column.

It was found that three main components were obtainable by column chromatography. The component 15 S naving the largest sedimentation rate and the component 2.5 S having smallest rate were revealed to be homogeneous, whereas the middle component was mixture of proteins probably produced by the dissociation of a protein compnent during column chromatography. The difficulty of the isolation of the intact molecule of the middle component would probably depend upon the instability of this protein. This seems likely to elucidate the variety of chromatographic diagram of the proteins contained in body fluid of the silk worm.

Studies on Flavonoid. Part III. Flavonoid in the Cocoon of Silkworm. (p. *977~980*)

By Keizo HAYASHIYA

(*School of Textile Fibers, Kyoto Technical Univresity*)

The autdor and co-workers, in the previous paper, pointed out that there were nine pigments in the green cocoon of silkworm, and five of them were flavonoids and the other were flavonoid-like pigments and that these pigments were in the state of glucosides in the cocoon and were constituted in the combination of four aglycones and glucose.

The author, as having shown in this paper, obtained two aglycones in the crystalline state and named temporarily them cocoon pigment I and II respectively. The properties of them were as follows:

	cocoon pigment I	cocoon pigment II
1. melting point	285°C. decomp.	194~5°C.
2. colour reaction with $FeCl_3$ solution	green-brown	green
3. // with $AlCl_3$	yellow	pale yellow
4. // with NaOH	yellow	red-violet
5. // with $Pb(CH_3CO_2)_2$	red-yellow	——
6. // with HCl and Mg	orange	yellow
7. molecular weight	317	——
8. assumed formular	$C_{15}H_{10}O_7$	——

The results from these tests indicated that the cocoon pigment I was a flavone derivative and the pigment II was flavone-like phenol derivative. This indication was

also supported with the ultra-violet and infra-red absorption spectra of these pigments.

Studies on Myrosinase. Part IV. Examinations on the Inhibition of Myrosinase. (p. *980~984*)

By Zenji NAGASHIMA and Masaaki UCHIYAMA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University*)

Experiments on the inhibition of myrosinase (white mustard) were conducted in order to examine the Neuberg's consideration and to study the destruction mechanism of mustard oil glucoside by the enzyme.

If the myrosinase is a mixture of myrosulfatase and thioglucosidase as the Neuber's opinion, some different effects of inhibiting agents must be exhibited on the two enzymes.

These two enzymes were inhibited by SH inhibitor (such as PCMB, monoiodoacetic acid) and other (Cu^{++}, Fe^{++}, Hg^{++}, KIO_3, I_2, hydroxylamine etc.), and remarkably activated by ascorbic acid. However, all these effects of inhibition and activation were always parallel in the two enzyme-reactions. Selective inhibitor or activator, specific on either of the two enzymes, could not be found.

From the facts thus obtained and described in the previous papers, myrosinase was confirmed to be a homogenous enzyme.

Hydroxylation of Steroid by *Syncephalastrum racemosum*. (Studies on the Hydroxylation of Steroid by Microorganisms. Part II.) (p. *985~990*)

By Toshinobu ASAI, Kō AIDA, Tokuji TANAKA, Eiji OHOKI and Takemasa MATSUHISA

(*The Institute of Applied Microbiology, University of Tokyo*);

Yoshito TAKEDA and Yasuji INUI

(*Institute of Sanraku Shuzo Co., Ltd.*)

Taxonomical studies of the strain reported in the preceeding paper to have a strong action to hydroxylate steriods were carried out. This strain was found to belong to the genus *Syncephalastrum* and accorded with the description of *S. racemosum* in respect to the dimensions of several organs and the number of spores contained in sporangia.

With this strain, the oxidative ability of various steroids were studied. Progesterone was oxidized to 7α, 15β-dihydroxprogesterone in a short period of incubation, but after long period it was transformed to 7α, 14α, 15β-trihydroxyprogesterone. When 11α-hydroxyprogesterone and Richstein's Compound S were used as substrates, 6β, 11α-dihydroxyprogesterone and 11-epihydrycortisone were produced, respectively. However, the fungs failed to hydroxylate 6β, 11α-dihydroxyprogesterone and hydrocrotisone even after long period of fermentation.

Biochemical Studies on the Growth of Sweetpotatoes. Part V. Changes of the Amylose Content, Blue Value, Iodine Starch Absorption Curve and Inorganic Constituents of the Starch Present in the Growing Sweetpotato Root. (p. *990~996*)

By Shigeo SUZUKI, Taro TAMURA, Yoshiro NEMOTO and Katsusuke ARAI

(*Food Research Institute, Ministry of Agriculture and Forestry, Tokyo, Japan*)

Samples of starch accumlated in the roots of two varieties of sweet potato, Norin No. 1 and Okinawa No. 100, have been isolated at intervals throughout the period of growth of the plants. Amylose content and blue value were higher at the earlier stage of growth and decreased in the middle of August but showed a constant value throughout the next growing period. The figures for the potassium and calcium contents of the starch showed no definite upward or downward trend, but phosphorus content showed definite upward trend in accordance with the growth. The phosphorus contents of Norin No. 1 sweet potato were higher than those of Okinawa No. 100 throughout all growing period.

(*continued from front cover*)

Microbiology and Fermentation Industry

Short Communications

Printed by
KASAI PUBLISHING & PRINTING CO.
(*PAN-PACIFIC PRESS*)
Minato-ku, Tokyo, Japan
Tels. (50) 1421-24